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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b>  A61K 37/00, C07C 241/00	<b>A1</b>	<b>(11) International Publication Number:</b> WO 93/14779  <b>(43) International Publication Date:</b> 5 August 1993 (05.08.93)
<b>(21) International Application Number:</b> PCT/US93/00906 <b>(22) International Filing Date:</b> 29 January 1993 (29.01.93)  <b>(30) Priority data:</b> 07/828,388                      30 January 1992 (30.01.92)      US 08/011,666                      29 January 1993 (29.01.93)      US  <b>(71) Applicant:</b> CORVAS INTERNATIONAL, INC. [US/US]; 3030 Science Park Road, San Diego, CA 92121 (US).  <b>(72) Inventors:</b> BRUNCK, Terence, Kevin ; 4949 Quincy, San Diego, CA 92109 (US). PEPE, Michael, Gary ; 1016 1/2 Loring Street, San Diego, CA 92109 (US). PEARSON, Daniel, Andrew ; 669 South Nardo, T-14, Solana Beach, CA 92075 (US). WEBB, Thomas, Roy ; 2250 Colony Terrace, Encinitas, CA 92024 (US).		<b>(74) Agents:</b> BIGGS, Suzanne, L. et al.; Lyon & Lyon, 611 West Sixth Street, 34th Floor, Los Angeles, CA 90017 (US).  <b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> TRYPSIN INHIBITORS  <b>(57) Abstract</b>  Novel compounds having activity against trypsin are disclosed. Specifically, novel peptide aldehyde analogues that have substantial potency and specificity as inhibitors of mammalian pancreatic trypsin are presented. The compounds are useful in the prevention and treatment of the tissue damage or destruction associated with pancreatitis.		

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DESCRIPTIONTrypsin InhibitorsRelated Applications

This application is a continuation-in-part of Brunck, Pepe and Webb, entitled "Trypsin Inhibitors", filed January 30, 1992, U.S. Serial No. 07/828,388, pending in the Patent Office and hereby incorporated by  
5 reference herein, including the drawings attached thereto.

Field of the Invention

This invention relates to inhibitors of trypsin useful for treatment of pancreatitis.

10

Background of the Invention

In a healthy mammal, the pancreas produces and secretes enzymes that digest carbohydrates, fats and proteins in the gastrointestinal tract. They are produced  
15 in inactive form (termed proenzyme) and subsequently are converted to an active form in the small intestine giving rise to a cascade of amylolytic, lipolytic and proteolytic activity. The cascade is thought to begin with the conversion of pancreatic trypsinogen to trypsin catalyzed  
20 by enterokinase, a proteolytic enzyme associated with the small intestine. The newly-formed trypsin then converts the other pancreatic proenzymes into their active forms to trigger a burst of enzymatic activity characterized as digestion. Greenberger et al., Diseases of the Pancreas,  
25 "Harrison's Principles of Internal Medicine," 11th Edition, p. 1372, McGraw-Hill, (E. Brunewald et al. edit, 1987).

In the absence of perturbing factors, the pancreas is able to protect itself from the autodigestion which  
30 could result from the digestive enzymes it produces. The pancreatic acinar cells (where the digestive enzymes are synthesized and stored) provide three control mechanisms to prevent their own destruction. First, the enzymes are

produced as catalytically inactive proenzymes. Second, after their synthesis, but before secretion into the digestive system, the enzymes are segregated from the acinar cell cytoplasm in lysosomes (membrane-bound intracellular organalles). Third, the lysosomes containing the enzymes contain potent protein inhibitors of trypsin which prevent premature activation of other hydrolytic proenzymes. Steer et al., New England Journal of Medicine, 316: 144 (1987).

10 In the presence of perturbing factors, a disorder of the pancreas termed pancreatitis (either acute or chronic) may result. Acute pancreatitis can manifest itself as a mild and self-limiting disorder (termed edematous pancreatitis), or in a more severe form (termed  
15 necrotizing pancreatitis) where permanent damage to pancreatic tissue occurs. Chronic pancreatitis results in extensive and permanent destruction of the pancreas. Greenberger et al., supra, at 1372.

Acute pancreatitis is usually associated with  
20 biliary tract stones, while chronic pancreatitis is often associated with chronic alcohol abuse. Steer et al., supra, at 144. Pancreatitis may also arise as a complication of cardiac surgery involving cardiopulmonary bypass procedures, and is reported to follow all types of  
25 open-heart surgery, including cardiac transplantation. Castillo et al., New England Journal of Medicine, 325: 382 (1991). Moreover, bouts of acute pancreatitis are occasionally induced following gastrointestinal diagnostic and surgical procedures, such as bile duct exploration,  
30 sphincteroplasty, distal gastrectomy, splenectomy, and endoscopic retrograde cholangiopancreatography. Bardenheier et al., Am. J. Surg. 116: 773 (1968); Cotton, Gut, 18: 316 (1977). Significant pancreatic injury has  
35 abdominal trauma which occasionally results in obstructive chronic pancreatitis.

Pancreatitis is characterized by damage to the pancreas and surrounding tissues which arises from

autodigestion of the cells by the various digestive enzymes activated by trypsin. Animal studies of chemically-induced pancreatitis suggest that the disorder is rooted in the inability of pancreatic acinar cells to excrete the digestive proenzymes. This results in the activation of trypsinogen to trypsin by lysosomal hydrolases within the cell, with the amount produced exceeding protective levels of protease inhibitor normally available. Steer et al., *supra*, at 148; Gabryelewicz, Digestion, 40: 19 (1988). This results in the subsequent activation of the other digestive enzymes co-localized with trypsin in the lysosome. These activated digestive enzymes cause edema, interstitial hemorrhage, vascular damage, coagulation necrosis, fat necrosis and parenchymal cell necrosis, Greenberger et al., *supra*, at 1372.

The activated digestive enzymes may subsequently enter the blood and the peritoneal cavity and can lead to secondary multiple organ damage. Although the blood contains trypsin inhibitors, it has been reported that trypsin complexed with one such inhibitor, alpha-2-macroglobulin, remains active. Rinderknecht et al., Biochim. Biophys. Acta, 295: 233 (1973); Harpel et al., J. Clin. Invest., 52: 2175 (1975); Rinderknecht et al., Biochim. Biophys. Acta, 377: 158 (1975). This active complex is thought to contribute in part to the metastatic proteolytic damage observed in pancreatitis. Jones, et al., Gut, 23: 939 (1982).

A number of compounds have been examined for treatment of pancreatitis. Specifically, aprotinin, Futhan, Foy, Foy-305 and the leupeptins.

Aprotinin is a polypeptide of 58 amino acids and is reported to be a potent inhibitor of trypsin, with a dissociation constant ( $K_d$ ) of  $3 \times 10^{-11}$  M. Jones, *supra*, at p. 939. However, it is also reported to be ineffective in the treatment of human acute pancreatitis. Imrie et al., Br. J. Surg., 65: 337 (1978); M.C.R. Working Party, Lancet, ii: 632 (1977); Niederau et al., *supra*, at 1061. Aprotinin is also an inhibitor of the coagulation factors,

kallikrein and plasmin with a  $K_d$  of  $1 \times 10^{-7}M$  and  $2 \times 10^{-10}M$  respectively. Kassell et al., Biochem. Biophys. Res. Commun., 18: 225 (1965); Fritz et al., Arzneim. Forsch. 33: 479 (1983); Lazdunski et al., Proteinase Inhibitors, p 420, Springer Verlag (H. Fritz et al. ed. 1974); Trautschold et al., Biochem. Pharmacol., 16: 59 (1967).

Futhan is a nonpeptidyl low molecular weight protease inhibitor first synthesized by Fuji et al., Biochim. Biophys. Acta, 661: 342 (1981). It is also known as nafamstat mesilate, FUT-175, and 6-amidino-2-naphthyl-4-guanidino benzoate dimethanesulfonate. It is reported to be effective in the treatment of acute pancreatitis induced in animal models. Iwaki et al., Jap. J. Pharmac. 41: 155 (1986); Gabryelwicz et al., supra, at p 22. It is a potent inhibitor of trypsin, as well as the coagulation enzymes, kallikrein, factor Xa, and thrombin. Aoyama et al., Japan J. Pharmacol., 35: 203 at 209 (1984); Hitomi et al., Hemostasis, 15: 164 (1985).

Foy (also known as gabexate mesilate) and Foy-305 (also known as camostate) are also nonpeptidyl low molecular weight protease inhibitors. Both are reported to be effective to varying degrees in the treatment of acute pancreatitis induced in animal models. Niederau, supra, at 1061; Lankisch et al., Gastroenterology, 96:193 (1989). Both compounds are reported to be effective inhibitors of trypsin, as well as the coagulation/fibrinolysis enzymes, kallikrein, thrombin, plasmin and C1r complement system enzyme. Muramutu et al., Biochim. Biophys. Acta, 268: 221 (1972); Takasugi et al., J. Med. Sci., 29: 188 (1980); Tamura et al., Biochim. Biophys. Acta, 484: 417 (1977).

The leupeptins are low molecular weight peptidyl aldehydes consisting of N-acetyl-L-leucyl-L-leucyl-L-argininal, N-propionyl-L-leucyl-L-leucyl-L-argininal and their analogs which contain L-isoleucine or L-valine in place of L-leucine. Aoyagi et al., "Structures and Activities of Protease Inhibitors of Microbial Origin", Proteases and Biological Control, pp. 429-454, Cold Spring

Harbor Laboratory Press (Reich et al. edit. 1975). They are reported to prolong the survival of rats in which acute pancreatitis has been induced. Jones, supra, at p. 939. They are potent inhibitors of trypsin and other serine proteases. Chi et al., J. Antibiotics, XLII: 1506 (1989).

Various derivatives of the leupeptins have been disclosed which are also potent inhibitors of trypsin. N-benzyloxycarbonyl-L-pyroglutamyl-L-leucyl-L-argininal was shown to be a potent inhibitor of trypsin (with an IC<sub>50</sub> about 7 times lower than that for N-acetyl-L-leucyl-L-leucyl-L-argininal) (Saino et al., J. Antibiotics, XL1: 220 (1988)).

U.K. Patent Application 2,153,825 and Japanese Application 60-163815 describe naphthalene derivatives of arginine as trypsin inhibitors useful for treatment of pancreatitis; and Niederau et al., Gastroenterology 88:1192 (1985) describe proglumide, benzotript and secretin as protective agents against caerulein-induced pancreatitis in mice.

#### Abbreviations

The following abbreviations are used in this application.

- "Bn" refers to benzyl.
- "Boc" refers to t-butoxycarbonyl.
- "BOP" refers to benzotriazol-1-yloxy-tris-(dimethylamino)- phosphonium-hexafluorophosphate.
- "CDI" refers to carbonyldiimidazole.
- "DCM" refers to dichloromethane.
- "DIEA" refers to diisopropylethylamine.
- "DMF" refers to N,N-dimethylformamide.
- "Fm" refers to 9-fluorenemethyl.
- "IPA" refers to isopropanol.
- "MeOH" refers to methanol.
- "NaOAc" refers to sodium acetate.
- "NMM" refers to 4-methylmorpholine.
- "Ph" refers to phenyl group.



"Ppa" refers to protected peptide or analog.

"TBS" refers to 0.1 M Tris, 0.14 M sodium chloride,  
pH 7.4.

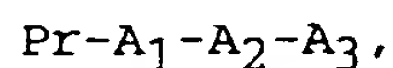
"TEA" refers to triethylamine.

5 "TFA" refers to trifluoroacetic acid.

"THF" refers to tetrahydrofuran.

#### Summary of the Invention

Applicants have discovered that compounds having the  
10 general structure:



where Pr is a hydrophobic group; A<sub>1</sub> is glutamic acid (Glu) or aspartic acid (Asp), or an equivalent of Glu or Asp; A<sub>2</sub> is proline (Pro) or an equivalent of Pro having a 4- or 5-  
15 membered ring structure; A<sub>3</sub> is Arginal (Arg-aldehyde or Arg-al) or an equivalent thereof are very specific and active trypsin inhibitors. In contrast to previously described trypsin inhibitors these compounds show potency and selectivity. That is, the described compounds have  
20 little to no inhibitory activity [i.e., have an IC<sub>50</sub> value substantially greater (i.e., more than ten fold greater) than that with trypsin] against one or more other serine proteases with physiologically significant activities, including those involved in blood clotting, e.g.,  
25 kallikrein, thrombin and the activated coagulation factors XII, XI, IX, VII, X, and II; serine proteases involved in clot dissolution e.g., plasmin, tissue plasminogen activator (tPA), and urokinase (UK); serine proteases involved in clot prevention e.g., Protein C; and serine  
30 proteases involved in complement mediated cell lysis, e.g., C1r and C1s. See, Colman et al., "Overview of Hemostasis" at pp 3-15, Bachmann, "Plasminogen activators", at pp 318-339, Owen, "Protein C", at 235-241 in Hemostasis and Thrombosis, Basic Principles and  
35 Clinical Practice, 2nd Edition, J.B. Lippincott Company (Colman et al. edit., 1987), and Eisen, "Complement", Microbiology, 3rd Edition, p. 456, Harper & Row (Davis et al., 1980). Because of their unexpected selectivity,



these compounds will be advantageous over other trypsin inhibitors known in the art because they will not have undesirable side effects resulting from inhibiting other useful and necessary protease activities in the body.

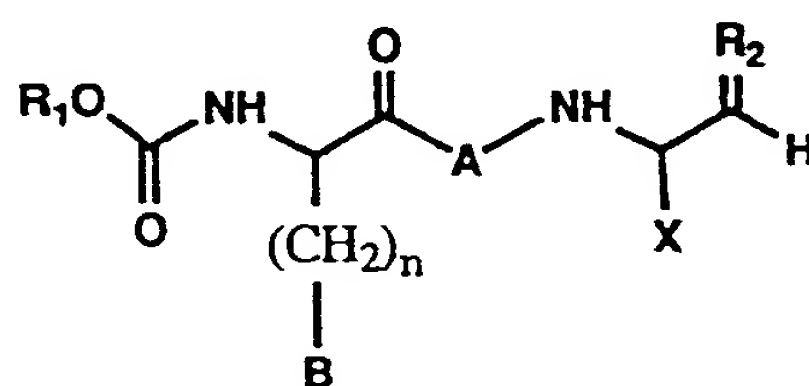
- 5 This property also permits the compounds to be administered intravenously and orally with few side effects.

By "equivalent" is meant to include variations in the general structure of one or more amino acids or  
10 hydrophobic groups which have little if any deleterious affect on the inhibitory activity of the compound compared to the use of the designated amino acid or hydrophobic group. Such variations are well known in the art and can be determined without undue experimentation. They include  
15 those variations in the general formula shown below. For example, the hydrophobic group is hydrophobic enough to provide a potent inhibitory activity. Arginine equivalents will function to direct the inhibitor to the active site of trypsin. Examples of such equivalents  
20 include an L-or D-isomer of argininal, homoargininal, guanidinoaminobutyral, guanidinoaminopropional, (Me<sub>2</sub>)Arg, (Et<sub>2</sub>)Arg, p-aminomethyl-phenylalaninal, p-amidinophenylalanine, p-guanidinophenylalanine, a conformationally constrained arginine analog as described  
25 by T.R. Webb and C. Eigenbrot, J. Org. Chem. 56:3009 (1991), or mono- or di-substituted N-alkyl derivative thereof wherein alkyl means a lower alkyl, preferably methyl. The Glu or Asp is a carboxylated non-cyclic amino acid and equivalents thereof. Such equivalents would  
30 include  $\gamma$ -R' esters of glutamic acid,  $\beta$ -R' esters of aspartic acid, or R'-substituted tetrazoles where the tetrazole substituted for the carboxylic acid group of Glu or Asp. R' in these equivalents is H, lower alkyl of 1 to 6 carbons, or aralkyl of about 6 to about 15 carbon atoms;  
35 and the Pro is a cyclic (preferably 4 or 5 membered ring) compound, not including a hydroxy group in the ring, examples include D- or L- isomers of proline,  $\beta$ -

methylproline,  $\beta,\beta$ -dimethylproline; dehydroproline, azetidine carboxylic acid.

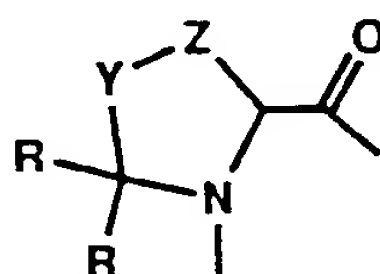
This invention also provides a pharmaceutical composition for the prevention and treatment of  
 5 pancreatitis, which includes one of the above compounds formulated as a pharmaceutically acceptable salt combined with a pharmaceutically acceptable carrier. The invention also provides a method for prevention of, or treatment of, pancreatitis.

10 Thus, in various aspects, the invention features novel peptide aldehydes, their pharmaceutically acceptable salts, and therapeutic compositions comprising these pharmaceutically acceptable salts in a suitable pharmaceutical diluent for use in the prevention and  
 15 treatment of pancreatitis, or other diseases characterized by an elevated level of trypsin activity. The novel compounds include those having the general formula:



(I)

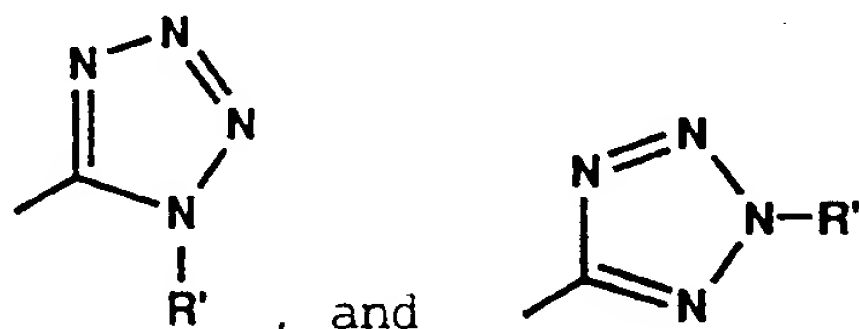
20 where  $\text{R}_1$  is a branched alkyl, a cyclic or polycyclic alkyl (which may be substituted with one or more alkyl groups, preferably of 1 to 5 carbon atoms) of 4 to 10 carbons,  $n$   
 25 is 1, 2 or 3; A is a group having the formula:



(II)

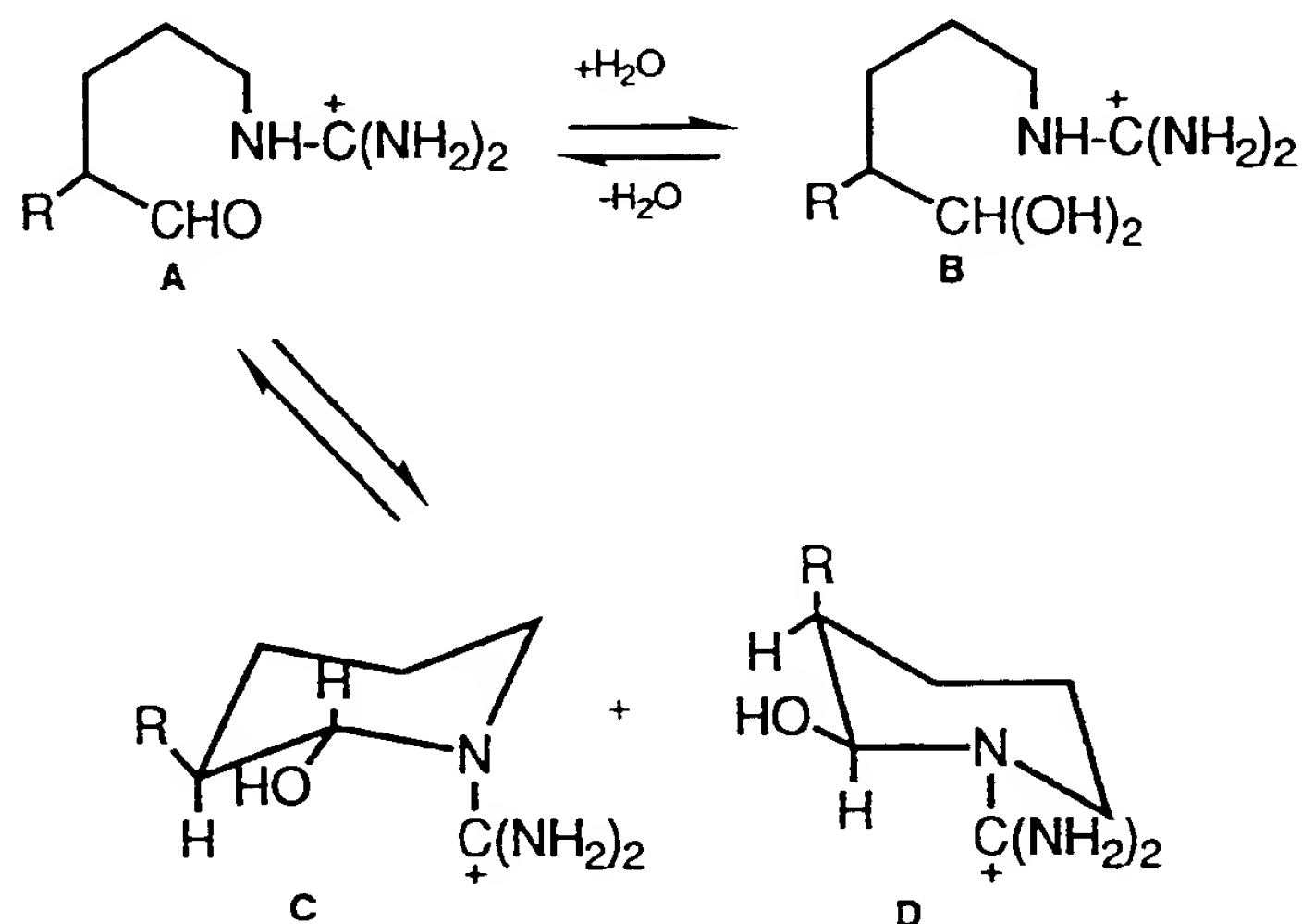
30 where Y, and Z are independently selected from a direct link, oxygen atom and methylene group where only one of Y and Z can be a direct link or an oxygen atom and each R is independently H or an alkyl group with 1 or 2 carbon

atoms; B is selected from a group consisting of  $-\text{CO}_2\text{H}$ ,  $-\text{CO}_2\text{R}'$ ,



wherein R' is as defined  
 5 above; X is  $-(\text{CH}_2)_3-\text{NH}-\text{C}(=\text{NH})-\text{NH}_2$ ,  $-(\text{CH}_2)_3-\text{C}(=\text{NH})-\text{NH}_2$ , 4-amidinophenylmethyl, 4-guanidinylphenylmethyl, or 4-aminomethylphenylmethyl, and their mono- and di-substituted N-alkyl derivatives, wherein the alkyl group is methyl, ethyl, propyl, isopropyl, butyl or isobutyl;  
 10 and R<sub>2</sub> is oxygen or  $\text{N}-\text{NR}_3-\text{C}(=\text{O})-\text{NR}_4$ , where R<sub>3</sub> is hydrogen, an alkyl group of 1 to 6 carbons, a phenyl group, an aralkyl group of 7 to 9 carbons, and R<sub>4</sub> is hydrogen, an alkyl group of 1 to 6 carbons, a phenyl group, an aralkyl group of 7 to 9 carbons, or a peptide or peptide analog,  
 15 provided that  $\text{N}-\text{NR}_3-\text{C}(=\text{O})-\text{NHR}_4$  is readily hydrolyzed at low pH to give the derivative with an oxygen atom. By such peptide analog derivatives having R<sub>2</sub> as  $\text{N}-\text{NR}_3-\text{C}(=\text{O})-\text{NHR}_4$  is meant to include prodrug forms of inhibitors of this invention which can be orally administered, and which  
 20 in the low pH (e.g., 6.0 or less of the stomach are cleaved to produce a potent trypsin inhibitor of the invention.

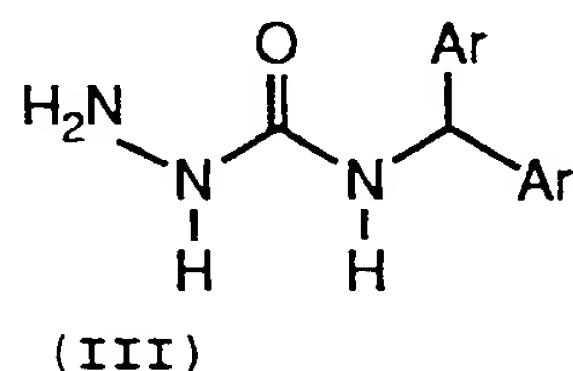
It is known that peptidyl arginine aldehydes exist in equilibrium structures in aqueous solutions. See,  
 25 Bajusz, S., et al., J. Med. Chem., 33:1729 (1990). These structures, as shown below, include the arginine aldehyde, A, aldehyde hydrate, B, and two amino cytol forms, C and D. The R group would represent the remainder of a given compound embodied in the present invention. The peptide  
 30 aldehydes of the present invention include within their definition all their equilibrium forms.



"Hydrophobic group" refers to a group which, when attached to molecules, would cause them to have an aversion to water and cluster together in order to avoid contact with water in an aqueous media. Typically, these include groups containing four or more carbon atoms, as a branched alkyl or alkenyl, and polycyclic alkyl substituents.

In preferred embodiments A is azetidine carboxylic acid, L-proline,  $\beta$ -methyl-L-proline,  $\beta,\beta$ -dimethyl-L-proline or 3,4-dehydro-L-proline.

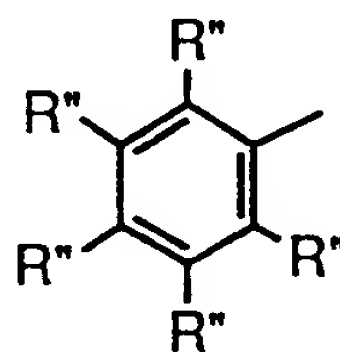
In another aspect, the invention features a method for synthesis of a peptide aldehyde. The method includes reacting a semicarbazide having the formula:



where Ar is optionally substituted phenyl or an equivalent thereof, with an N-protected amino acid aldehyde to produce a protected semi-carbazone, deprotecting the N-terminus of the protected semi-carbazone, and reacting the

deprotected semi-carbazone with an N-protected amino acid to produce an N-protected peptide.

In preferred embodiments, the Ar is phenyl; the amino aldehyde is chosen from the group consisting of argininal and lysinal; the deprotecting and reacting the deprotected semi-carbazone steps are repeated a plurality of times with either the same N-protected amino acid or a different N-protected amino acid; the Ar has the formula:



(IV)

where each R'' is selected independently from the group consisting of hydrogen, methyl, methoxy, halogen, ethyl and ethoxy.

In yet another aspect, the invention features a protected semi-carbazone formed by reacting a semicarbazide having the formula III above with an  $\alpha$ -N-protected amino aldehyde to produce a protected semi-carbazone.

Examples of methods useful for making these semi-carbazides and resulting semi-carbazones are described in Webb, "Reagents for Automated Synthesis of Peptide Analogues, U.S. Serial No. 07/627,753, filed December 14, 1990 assigned to the same assignee as the present invention, and hereby incorporated by reference herein. See also, Murphy et al., J. Am. Chem. Soc. 114:3156-3157 (1992).

#### Brief Description of the Drawing

Fig. 1 is a scheme illustrating a process for making a solid phase reagent which is subsequently used to make one or more compounds of the present invention, wherein "Bn" refers to benzyl, "t-Bu" refers to t-butyl, and "Boc" refers to t-butoxycarbonyl.

Fig. 2 is a scheme illustrating a process for synthesis of compound 21, wherein "i" refers to pTsoH/FmOH, toluene/reflux, "ii" refers to Boc-Asp- $\beta$ -benzyl ester/BOP/MM/DMF and "iii" refers to triethylamine/reflux.

Fig. 3 is a scheme illustrating a process for synthesis of compound 24, wherein "i" refers to CDI/13 followed by 14, "ii" refers to TFA/DCM, "iii" refers to 1/sodium acetate, "iv" refers to a protected peptide or analog as free acid (Ppa), e.g., 21 of Figure 2/BOP/MM/DMF, "v" refers to H<sub>2</sub>/Pd, and "vi" refers to H<sub>3</sub>O<sup>+</sup>.

#### Detailed Description of the Preferred Embodiments

##### 15 Trypsin Inhibitors

Inhibitors of this invention are generally described above. Below are provided examples of such inhibitors. These examples are not limiting in the invention; those in the art will readily recognize that equivalent inhibitors are synthesized by similar methods to those provided below. For example, one preferred embodiment is the compound, t-butyloxycarbonyl-L-aspartyl-L-prolinyl-L-argininal, which is both a potent and specific inhibitor of trypsin. The inhibitor constant (K<sub>i</sub>) for this compound against trypsin is 4.5 x 10<sup>-10</sup>M. Moreover, surprisingly little or no inhibition of other serine proteases was found (see Table 2, *infra*). Another preferred embodiment is the compound of Example 8 which is advantageously specific for inhibiting trypsin.

The specificity of compounds of this invention is unprecedented in the art. For example, McConnell et al., J. Med. Chem., 33:86 (1989) and Bajusz, Symposia Biologica Hungarica, 25:277 (1984) report that substitution of the N-terminal acetyl group or hydrogen of leupeptin with a more hydrophobic group, e.g., benzyloxycarbonyl (Cbz) or t-butyloxycarbonyl (Boc) has little effect on trypsin inhibitory activity and specificity or even results in a less potent and nonspecific trypsin inhibitor. Compounds

of this invention are generally at least one hundred (100) fold and up to one thousand (1,000) fold or greater, more potent inhibitors of trypsin than the known leupeptin analogs, and show substantially greater specificity.

5

### Synthesis

Compounds of the present invention may be synthesized by either solid or liquid phase methods. The functional groups of the amino acid derivatives used in such syntheses are protected by blocking groups, as described herein, to prevent undesired side-reactions during the coupling procedure. The solution-phase starting materials used are readily available from commercial chemical vendors including Aldrich, Sigma, Nova Biochemicals and the like.

The peptide aldehydes can be synthesized by sequential chemical attachment of amino acid derivatives using the solid phase synthesis reagents and methods disclosed by Webb, U.S. Patent Application, Serial No. 627,753, filed December 14, 1990, entitled "Reagents for Automated Synthesis of Peptide Analogues," assigned to the same assignee as the present invention, the disclosure of which is incorporated herein by reference. Fig. 1 illustrates the synthesis of a solid phase reagent to which subsequent amino acid derivatives are attached, details of which are provided in the examples, infra.

The present invention also features semicarbazone derivatives discussed above, prepared from the above peptide aldehydes. The semicarbazones are derivatives of the peptide aldehydes which protect the aldehyde functionality of the peptide aldehyde. Unlike the compounds described by McConnell et al., supra, at p. 88, such semicarbazone derivatives are soluble in organic solvents, are crystalline, and couple in high yield, which makes them useful for efficient synthesis of desired peptide aldehydes.

There have been reports of various methods for the solution synthesis of peptide aldehydes (see Bajusz et



al., J. Med. Chem. 33:1729-1735 (1990); McConnell et al., J. Med. Chem. 33: 86-93 (1990) and references cited therein; Kawamura et al., Chem. Pharm. Bull., 17;1902 (1969); Someno et al., *ibid*, 34, 1748 (1986); Westerik and  
5 Wolfenden, J. Biol. Chem., 247: 8195 (1972); and Ito et al., Chem. Pharm. Bull. 23, 3081, (1975)). McConnell et al., *supra* have used the unsubstituted semicarbazide as an aldehyde protecting reagent for the solution synthesis of peptide aldehydes. Galpin et al., Pept. Struct. Funct.,  
10 Proc. Am. Pept. Symp., 9th, 799-802 (Edited by: Deber, C., M., Hruby, V., J., Kopple, K., D., Pierce Chem. Co., Rockford, Ill.) have reported on the use of a soluble semicarbazide functionalized polymer which they have used for the manual preparation of some peptide aldehydes.

15 The methods cited above have significant limitations in scope and practical utility. Only a few of these methods have been shown to be applicable to the synthesis of peptide argininals. The procedures that use lithium aluminum hydride to generate the peptide aldehyde at a  
20 late stage in the synthesis (see Bajusz et al., *supra*) are not applicable to the synthesis of derivatives containing ester protecting groups or other functional groups sensitive to lithium aluminum hydride ( $\text{LiAlH}_4$ ). Therefore the hydride procedure is not suited for the synthesis of  
25 derivatives containing, for example, aspartic acid or glutamic acid when this reaction sequence is used. Procedures that use the unsubstituted semicarbazide group as a protecting group for the argininal (see McConnell et al., cited above) suffer from low yields and significant  
30 solubility problems. The procedures for the solid phase automated synthesis of peptide aldehyde analogs described in the commonly assigned U.S. Patent Application, Serial No. 07/627,753, filed December 14, 1990, has overcome many of these problems. However, under certain circumstances,  
35 for example, in the case of large scale synthesis, due to cost considerations, the use of the solution phase methods described herein may be particularly advantageous.

We have therefore devised a new protecting group that has many advantages over the existing aldehyde protecting group. The procedure for the synthesis of this protecting group (the 4-diphenylmethyl-semicarbazide group or DPS group) and its use for the preparation of peptide aldehydes, is illustrated in Figure 3. The DPS group has many advantages over the simple semicarbazide group; including the much greater solubility of the resulting DPS semicarbazone derivatives; also, many of the intermediates are crystalline and can be purified by simple recrystallization. The intermediates also give good yields on coupling and deprotection, etc.

The commercially available 4-phenylsemicarbazide was also investigated by us as a potential aldehyde protecting group reagent. Using this reagent some protected argininals were converted to the corresponding 4-phenylsemicarbazones. Although these derivatives do not have sufficient solubility to be a practical alternative to the DPS derivatives, the 4-phenylsemicarbazones do offer some advantages over the simple semicarbazones.

#### Formulations

The present invention also includes the pharmaceutically acceptable salts of the compounds disclosed. These salts include acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid; other suitable acid addition salts are considered to be within the scope of this invention.

The present invention also includes compositions prepared for storage or administration which include a pharmaceutically effective amount of the disclosed compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985) hereby incorporated by reference herein. For example,

preservatives, stabilizers, dyes and even flavoring agents may be provided. Id. at 1449. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be  
5 used. Id.

A pharmaceutically effective dose of the composition required for the prevention or treatment of pancreatitis will depend on the route of administration, the type of mammal being treated, and the physical characteristics of  
10 the specific mammal under consideration. The compositions of the present invention may be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile  
15 solutions, suspensions for injectable administration, and the like. The dose and method of administration can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100  
20 mg/kg body weight/day is administered dependent upon potency of the inhibitor.

The potency and specificity of the compounds of the present invention are determined by in vitro assay methods well known in the art. Potency is assessed from the  
25 concentration of compound required to substantially inhibit the enzymatic action of trypsin. Specifically, the inhibitor constant,  $K_i$ , of the compounds can be determined by the method of Dixon, Biochem. J. 55:170 (1953). The specificity is assessed by determination of  
30 the concentration,  $IC_{50}$ , of compound required to give 50% inhibition of the enzymatic activity of the coagulation enzymes, kallikrein, factor XIa, factor VIIa, factor Xa, thrombin; the fibrinolysis enzymes, plasmin, tissue plasminogen activator (tPA), and urokinase (UK), and the  
35 anticoagulation enzyme, protein C. Specificity is found when the concentration of compound giving 50% inhibition of trypsin is low relative to the concentration required to give like inhibition of the other enzymes. That is,

the  $IC_{50}$  for trypsin should be less than about 0.1, preferably 0.01  $\mu M$ , while the  $IC_{50}$  for the other enzymes is at least 10-1000 fold (preferably 10-100 fold) greater, e.g., greater than 1  $\mu M$ .

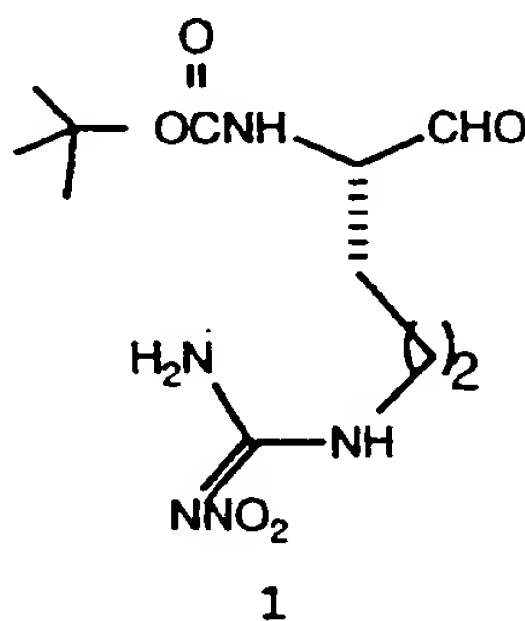
5 The efficacy of the compounds of the present invention as a prophylactic treatment for pancreatitis is assessed in vivo using the chemically-induced animal model of Niederau et al., Gastroenterology 88:1192-1204 (1985).

10 The compounds of the present invention, as selected by the in vitro and in vivo methods disclosed, are potent and highly specific inhibitors of trypsin and thus are useful for the prevention and treatment of pancreatitis in mammals.

15 The invention will now be further illustrated by the following examples. Unless otherwise specified, the procedures described in the following examples are conducted at ambient temperature and pressure. The first seven examples are illustrated in Fig. 1.

20 Example 1:

Preparation of  $\alpha$ -N-t-butoxycarbonyl- $N^G$ -nitro-argininal



25 The following procedure for the synthesis of alpha-t-butoxycarbonyl- $N^G$ -nitro-argininal 1 is an example of a general procedure for the preparation of Boc-amino acid aldehydes. See Patel et al., Biochim. Biophys. Acta, 748, 321-330 (1983). 12.7 g Boc- $N^G$ -nitro-arginine (40 mmols) and 7.0 g carbonyldiimidazole (CDI; 43 mmols) were added  
30 at room temperature (between 20 and 25°C) to 200 mL dry tetrahydrofuran (THF) and allowed to stir for 30 minutes. The reaction mixture was cooled to -78°C and 35 mL  $LiAlH_4$

(1 M in THF) was added dropwise over thirty minutes. The reaction was allowed to stir for an additional 1 hour at -78°C. Next, 18 mL acetone was added and the mixture quickly added to 400 mL 1N HCl. The mixture was extracted  
5 twice with 100 mL ethyl acetate. The ethyl acetate washes were combined and then washed two times each with 100 mL water, 100 mL saturated NaHCO<sub>3</sub> and 100 mL saturated NaCl (brine). The solution was dried using MgSO<sub>4</sub> and concentrated to a foam. The crude weight of the alpha-t-  
10 butoxycarbonyl-N<sup>G</sup>-nitro-argininal was 6.36 g (21 mmole; yield 52%).

The following alternative procedure for the synthesis of alpha-t-butoxycarbonyl-N<sup>G</sup>-nitro-argininal 1 is a modification of the procedure of Fehrentz and Castro,  
15 Synthesis, 676 (1983).

Boc-N<sup>G</sup>-nitro-arginine was obtained from Calbiochem. N-methyl piperidine, N,O-dimethylhydroxylamine hydrochloride and isobutylchloroformate, and lithium aluminum hydride were obtained from Aldrich Chemical  
20 Company, Inc. Dichloromethane, ethyl acetate, methanol and tetrahydrofuran may be obtained from Fisher Scientific Company.

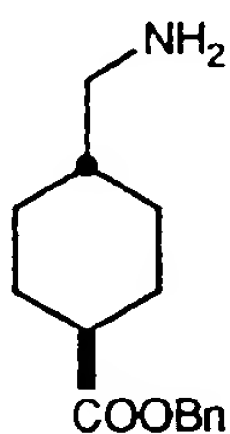
11.4 mL N-methyl piperidine was slowly added to a stirred suspension of 8.42g (94 mmole) N,O-  
25 dimethylhydroxylamine in 75 mL dichloromethane which had been cooled to about 0°C. The solution was allowed to stir for 20 minutes to give the free hydroxylamine, and then was kept cold for use in the next step.

In a separate flask, 30.0 g (94 mmole) Boc-N<sup>G</sup>-  
30 nitroarginine was dissolved by heating in about 1400 mL tetrahydrofuran and cooled under nitrogen to 0°C. 11.4 mL N-methylpiperidine and 12.14 mL (94 mmole) isobutylchloroformate was added and the mixture stirred for 10 minutes. The free hydroxylamine prepared above was  
35 added all at once, the reaction mixture allowed to warm to room temperature, and then stirred overnight.

The resulting precipitate was filtered off, then washed with 200 mL tetrahydrofuran. After concentrating

the filtrates to about 150 mL under vacuum, 200 mL ethyl acetate was added, followed by ice to cool the solution. The cooled solution was washed with two 75 mL portions of 0.2 N hydrochloric acid, two 75 mL portions 0.5N sodium hydroxide, and one 75 mL portion brine, and then dried over anhydrous magnesium sulfate. Upon concentration in vacuum, 22.7 g (70% yield) of solid Boc-N<sup>G</sup>-nitro-arginine N-methyl-O-methylcarboxamide was recovered. Thin layer chromatographic analysis in 9:1 dichloromethane/methanol (silica gel) showed one spot.

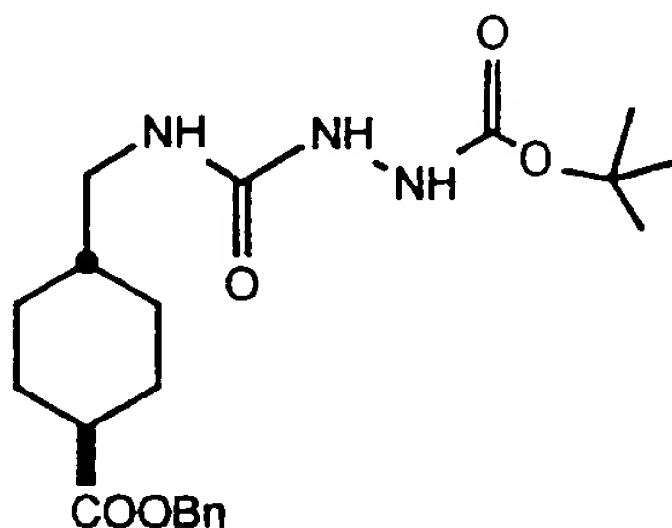
A flask was placed under a nitrogen atmosphere and cooled to -50°C, then charged with 70 mL (70 mmole) 1 N lithium aluminum hydride (in tetrahydrofuran) and 500 mL dry tetrahydrofuran. 50mL of a solution containing 66 mmole Boc-N<sup>G</sup>-nitroarginine N-methyl-O-methylcarboxamide in dry tetrahydrofuran was slowly added while the temperature of the reaction mixture was maintained at -50°C. After allowing the reaction mixture to warm to 0°C by removal of the cooling, it was recooled to -30°C, at which temperature 100 mL (0.2 mole) 2 N potassium bisulfate was added with stirring over about a 10 to 15 minute period. The reaction mixture was then allowed to stir at room temperature for 2 hours. After filtering off the precipitate, the filtrate was concentrated to 100 mL under vacuum. The concentrate was poured into 800 mL ethyl acetate, then was washed successively with two 50 mL portions 1N hydrochloric acid, two 50 mL portions saturated sodium bicarbonate, one 50mL portion brine. The combined aqueous extracts were extracted with 3-100 mL portions of ethyl acetate. All of the ethyl acetate washes were combined and then dried over anhydrous magnesium sulfate. The mixture was concentrated under vacuum to yield 18.5 g (95%) compound 1.

Example 2:Preparation of trans-4-(aminomethyl)-cyclohexane  
carboxylic acid benzyl ester para-toluenesulfonate salt

2

50 g (0.318 moles) trans-4-(aminomethyl)-cyclohexane  
carboxylic acid, 61.7 g (0.324 moles) p-toluenesulfonic  
acid, 250 mL (2.4 moles) benzyl alcohol, and 250 mL  
10 toluene were combined and stirred at room temperature.  
The mixture was refluxed for 24 hours and the liberated  
water removed azeotropically by means of a Dean and Stark  
apparatus. A clear solution was obtained after 5 hours of  
refluxing. The solution was allowed to cool to room  
15 temperature and the product crystallized. The mixture was  
vacuum filtered, washed with ether and dried in a vacuum  
oven to give 128.12 g (96% yield).  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  1.05  
(m, 2H), 1.43 (m, 2H), 1.59 (m, 1H) 1.85 (m, 2H), 2.03 (m,  
2H), 2.33 (m, 1H), 2.35 (s, 3H), 2.75 (d, 2H), 5.09 (s,  
20 2H), 7.23 (d, 2H), 7.32 (m, 5H), 7.69 (d, 2H). M.P. 154-  
156°C. See, Greenstein and Winitz, *Chemistry of the Amino  
Acids*. 2:942 (1986).



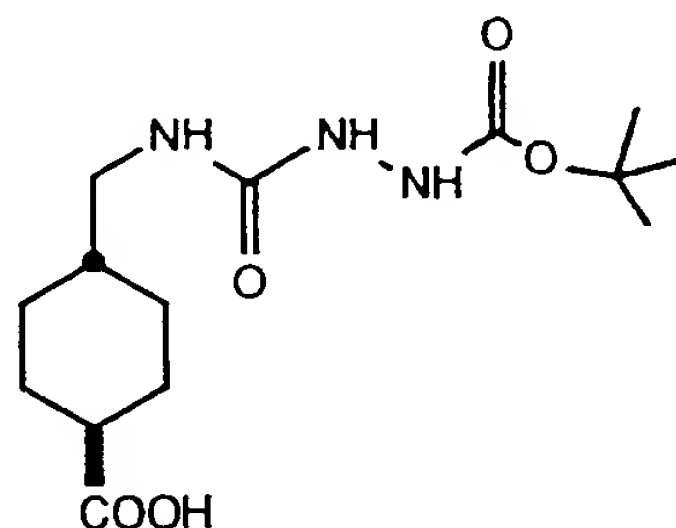
Example 3:Preparation of 1-t-butoxycarbonyl-semicarbazidyl-trans-4-methyl cyclohexane carboxylic acid benzyl ester

5

3

3.24 g (0.02 moles) carbonyldiimidazole (CDI) was dissolved in 45 mL of dimethylformamide (DMF) at room temperature under nitrogen. A solution of 2.48 g (0.02 moles) t-butyl carbazate in 45 mL DMF was added dropwise. 8.38 g (0.02 moles) solid benzyl ester 2 was added, followed by the dropwise addition of 3.06 mL triethylamine (TEA) over a 30 minute period. The reaction was allowed to stir at room temperature under nitrogen for one hour. Water (100 mL) was added and the mixture extracted three times with 50 mL ethyl acetate. The ethyl acetate layers were combined and extracted two times each with 75 mL 1N HCl, H<sub>2</sub>O, NaHCO<sub>3</sub>, NaCl and dried with MgSO<sub>4</sub>. The mixture was filtered and the solution was concentrated to give an oil. This material could be purified by recrystallization from ethyl acetate/hexanes (M.P. = 106-108°C) or used directly in the next step. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 0.94 (m, 2H), 1.42 (m, 2H), 1.45 (s, 9H), 1.81 (m, 2H), 2.02 (m, 2H), 2.27 (m, 1H), 3.17 (t, 2H), 5.09 (s, 2H), 5.51 (t, 1H), 6.46 (s, 2H), 7.34 (m, 4H).

25

Example 4:Preparation of 1-(t-butoxycarbonyl)-3-semicarbazidyl-trans-4-methyl-cyclohexane carboxylic acid

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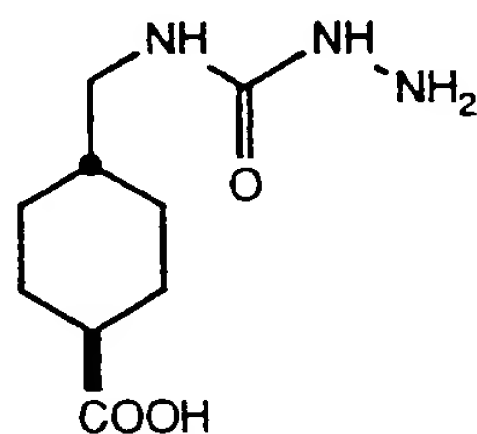
4

To the crude Boc-benzyl ester 3 from Example 3 above, 250 mL of methanol (MeOH) and 500 mg of 10% palladium on activated carbon were added. After shaking on the hydrogenator for one hour at 5 psig H<sub>2</sub>, the mixture was filtered with Celite through a fine fritted filter. The solution was concentrated to a foam, methylene chloride added, and a precipitate formed. The mixture was kept at 5°C for 65 hours. The crystallized material was filtered with ether and 4.0 g of crude product obtained (12.7 mmol; yield 62% overall yield from compound 2.) <sup>1</sup>H NMR (CD<sub>3</sub>OD), δ 0.96, (m, 2H), 1.42 (m, 2H), 1.46 (s, 9H), 1.82 (m, 2H), 1.97 (m, 2H), 2.18 (m, 1H), 3.0 (t, 2H). M.P. = 185-189°C.

20

Example 5:Preparation of semicarbazidyl-trans-4-methylcyclohexane carboxylic acid trifluoroacetate salt

25



5

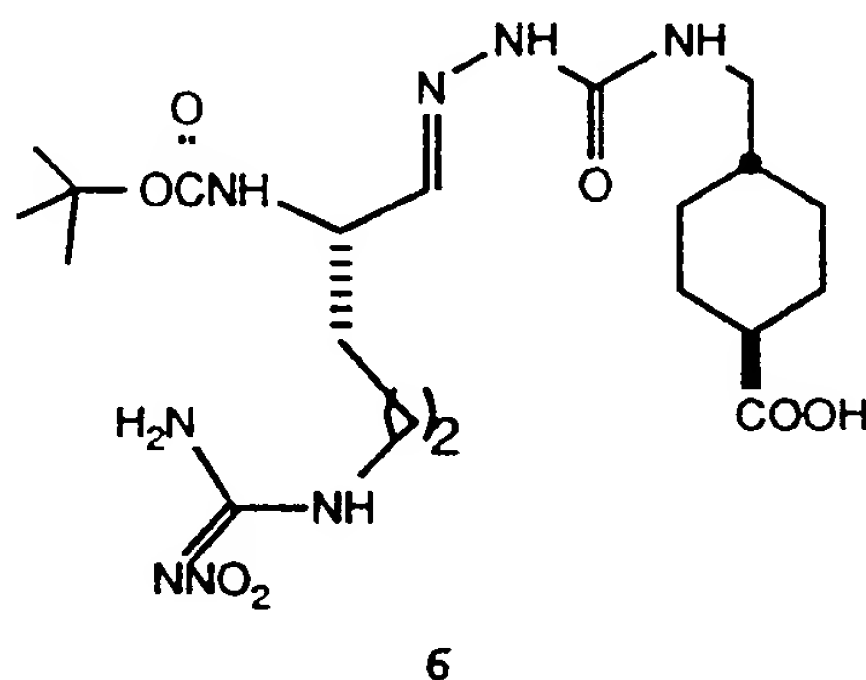
315 mg (1 mmol) of compound 4 was added to 10 mL trifluoroacetic acid (TFA) at 0°C and the resulting

solution stirred for 30 min. After this time the solution was added dropwise to 75 mL ether. A precipitate formed, and the mixture was filtered and washed with ether.

Weight of crude product was 254 mg, 0.77 mmoles; yield (77%).  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ),  $\delta$  1.0 (m, 2H), 1.38 (m, 2H), 1.43 (m, 1H), 1.84 (m, 2H), 2.01 (m, 2H), 2.22 (m, 1H), 3.04 (d, 2H). M.P. = 154-156°C.

Example 6:

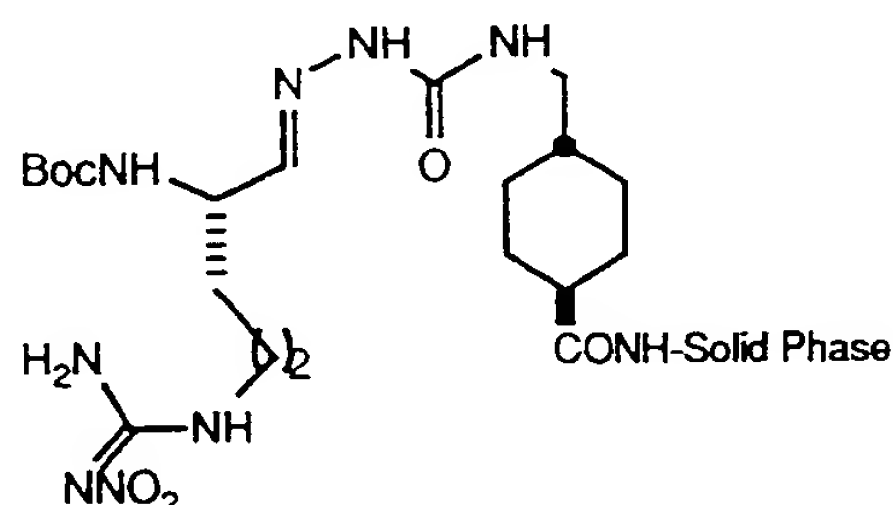
10 Preparation of  $\alpha$ -(t-butoxycarbonyl)-N<sup>9</sup>-nitro argininal-semicarbazonyl-trans-4-methylcyclohexane carboxylic acid



15

A solution of 13.7 g (41.6 mmoles) compound 5, 18.0 g (~59 mmoles) crude compound 1 in 135 mL ethanol containing 45 mL water, was treated with 9.41 g (69 mmoles) NaOAc and refluxed for one hour. This solution was allowed to cool and then poured into 0.1 N HCl and extracted three times with ethyl acetate. The combined organic phase was washed with water, then brine, dried with  $\text{MgSO}_4$  and concentrated to a small volume. This cloudy mixture was allowed to set overnight at 5°C to precipitate the product, which was isolated by filtration and dried under vacuum. This gave 9.9 g, 47% yield based on 5.  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ),  $\delta$  1.0 (m, 2H), 1.43 (s, 9H), 1.45-2.20 (m, 13H), 3.09 (d, 2H), 3.30 (m, 2H), 4.18 (bs, 1H), 7.10 (d, 1H). M.P. = 162-163°C.

30

Example 7:Synthesis of Semicarbazide Solid Support

5

7

Solid phase reagent 7 was prepared by placing 0.8 g (0.5 mmol, 0.62 g/mol) methyl-benzhydrylamine (MBHA) resin in a reaction vessel and washing 1 time with dichloromethane (DCM) (all washes require 10 mL of solvent with agitation for 1-2 minutes), 3 times with dimethylformamide (DMF), 2 times with 10% diisopropylethylamine (DIEA)/DMF, and 4 times with DMF. 5 mL DMF, 1 mmol 4-methylmorpholine (NMM) (102  $\mu$ l), 1 mmol benzotriazol-1-yloxy-tris-(dimethylamino)-phosphonium-hexa-fluorophosphate (BOP reagent) (443 mg), and 1 mmol compound 6 (500 mg) was added, mixed on a rotating wheel for 16 hours, and washed 3 times with DMF, 2 times with 10% DIEA/DMF and 3 times with DMF. The resin was then washed successively with DCM, methanol, and ether.

The resulting resin 7 shows 98-99% coupling yield by ninhydrin.

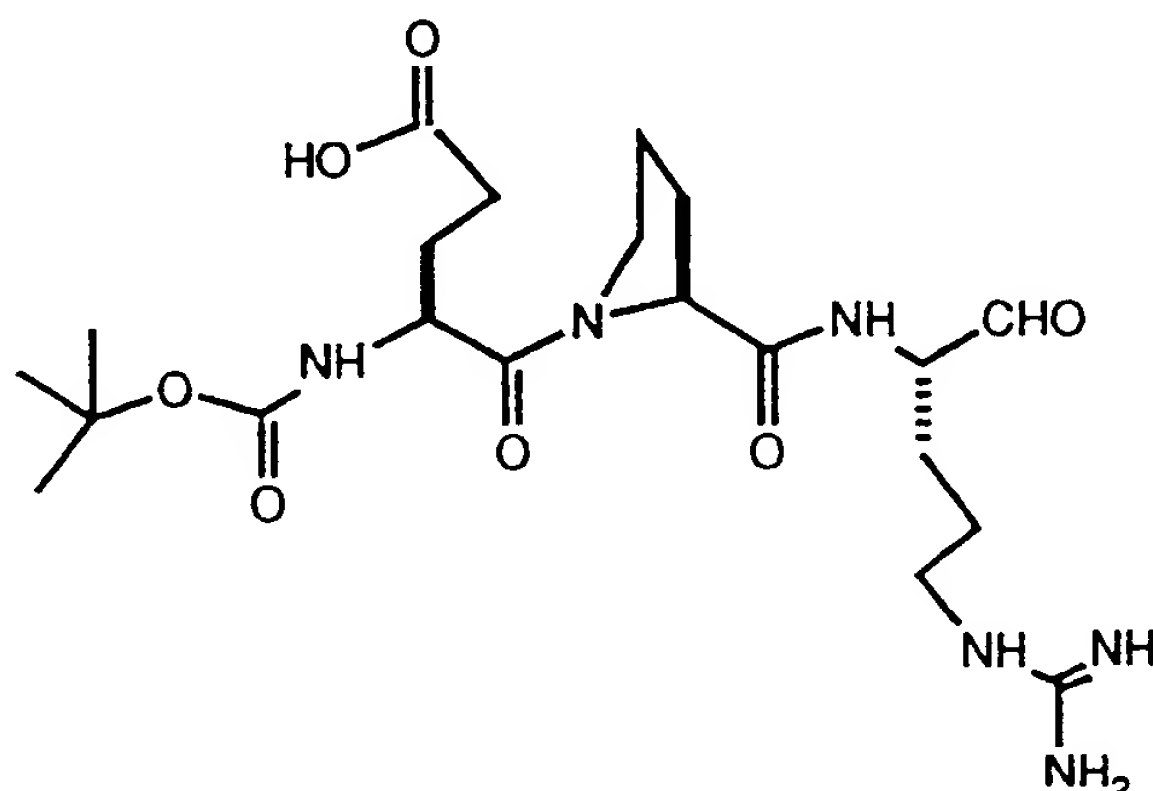
This resin was then extended at the N-terminus, with amino acids or amino acid analogs, on a conventional peptide synthesizer using standard t-Boc methodology as shown in the examples which follow.

The automated synthesis of peptide aldehydes was performed on an Applied Biosystems model 430A peptide synthesizer using the t-Boc chemistry conditions in the 430A user's manual. The resulting protected peptide aldehyde can be cleaved from support with formaldehyde and deprotected with hydrogen/Pd. The nitro group can be

removed from the guanidine group without reduction of the aldehyde.

Example 8:

5 Preparation of N-t-butyloxycarbonyl-L-Glu-L-Pro-L-argininal



10

The peptide aldehyde 8 was synthesized using an Applied Biosystems Model 430A peptide synthesizer as discussed above. The t-Boc chemistry conditions utilized were as provided in the instrument user's manual.

15 0.500 g resin 7 was made ready for use by removing the t-Boc protecting groups by treatment with 50% trifluoroacetic acid (in dichloromethane). After washing and neutralizing the acidity by treatment with 10% diisopropylethylamine (in dichloromethane), commercially  
20 available t-Boc-protected amino acids were coupled to the support reagent (and the growing amino acid support chain) in a sequential manner.

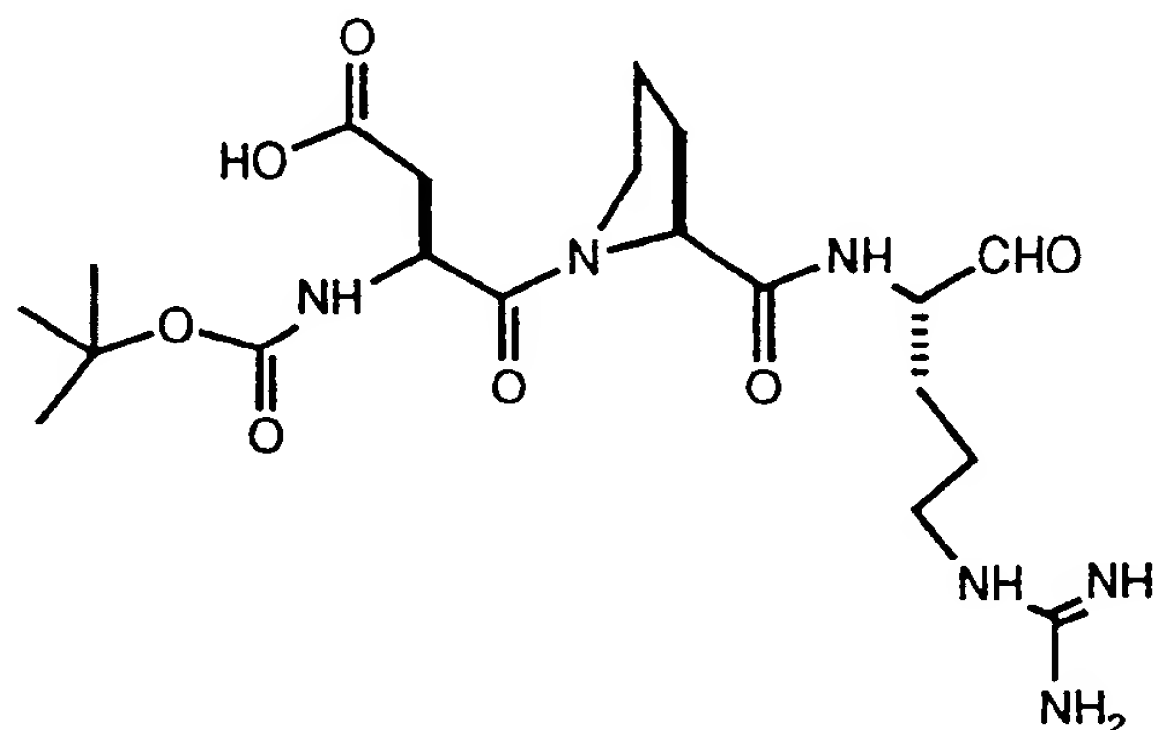
Thus, N-Boc-L-proline was attached to the resin using dicyclohexylcarbodiimide and 1-hydroxybenztriazole  
25 in dimethylformamide, followed by treatment with 50% trifluoroacetic acid (in dichloromethane) to remove the tBoc protecting group, a wash step and a wash with 10% diisopropylethylamine (in dichloromethane) to neutralize acidity. N-Boc-L-glutamic acid-γ-benzyl ester was coupled

in the same manner, except that treatment with 50% trifluoroacetic acid was omitted.

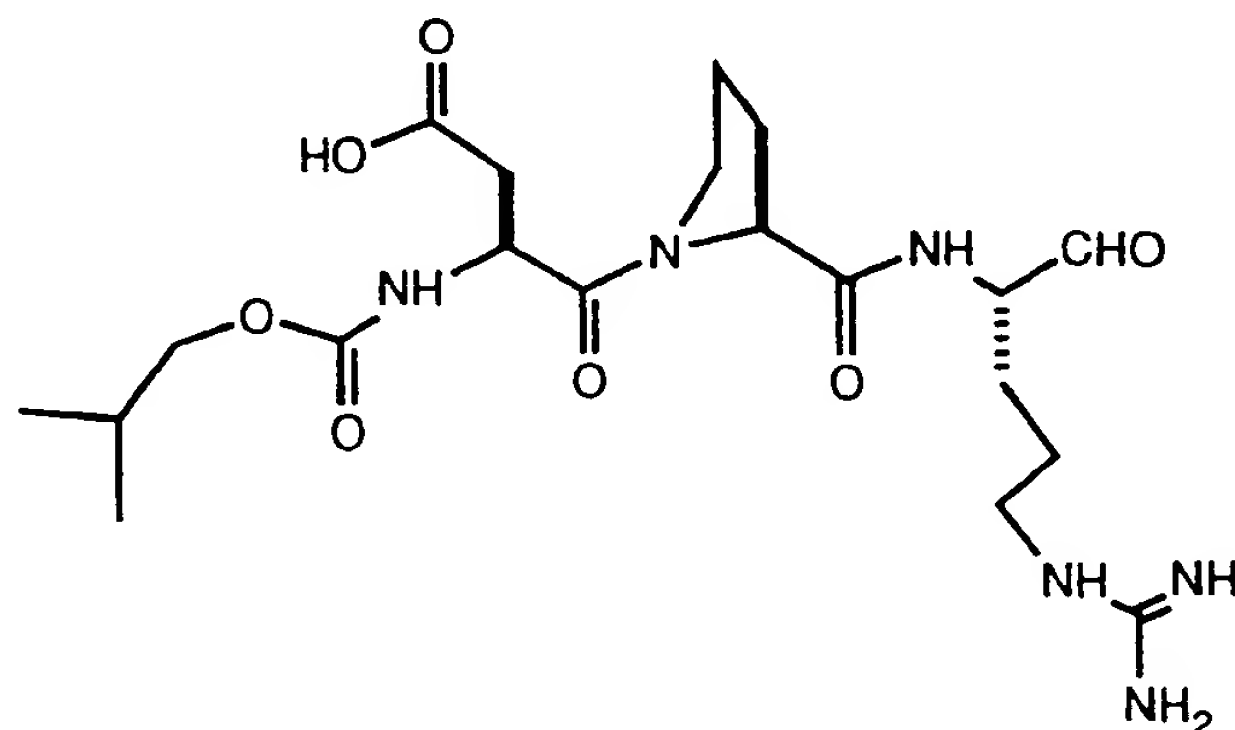
The peptide aldehyde was removed from the solid phase by treatment with a mixture of 5 mL tetrahydrofuran, 1 mL acetic acid, 1 mL formaldehyde and 0.100 mL 1 N HCl for 1 hour with stirring. After filtering this mixture, the resin was washed with 10 mL of tetrahydrofuran. The combined filtrates were diluted with 100 mL water and extracted with ethyl acetate. The ethyl acetate phase was then washed with saturated NaCl, dried over magnesium sulfate, and concentrated under vacuum.

To remove the nitro and benzyl protecting groups of the peptide aldehyde, the concentrated peptide aldehyde was taken up in a mixture comprising 10 mL of 10% water in methanol, 0.300 mL 1N HCl and 0.200 g palladium on carbon, and then treated with hydrogen at 5 psi for 45 minutes. The mixture was filtered through a fine fritted filter with Celite, washed with 10% water in methanol, and concentrated to give the crude peptide aldehyde.

The resulting peptide aldehyde was then purified using reverse phase HPLC on a 10 micron particle size, 300 angstrom pore size C-18 column, eluting with a water-acetonitrile (both containing 0.1% trifluoroacetic acid) gradient, where the gradient ran from 5% to 40% acetonitrile. The column fractions were analyzed by analytical HPLC and fractions containing pure product were pooled and lyophilized to yield product 8. Fast atom bombardment mass spectrometry gave observed molecular weight of 484 a.m.u.; calculated molecular weight was 484 a.m.u.

Example 9:Preparation of N-t-butyloxycarbonyl-L-Asp-L-Pro-L-argininal

Peptide aldehyde 9 was synthesized and purified in the same manner as described in Example 8. Here, N-Boc-L-proline was first attached to resin 7 followed by N-Boc-L-aspartic acid-beta-benzyl ester (in the place of N-Boc-L-glutamic acid-γ-benzyl ester). Again, treatment with 50% trifluoroacetic acid was omitted after the last coupling. Fast atom bombardment mass spectrometry gave observed molecular weight of 470 a.m.u.; calculated molecular weight was 470 a.m.u.

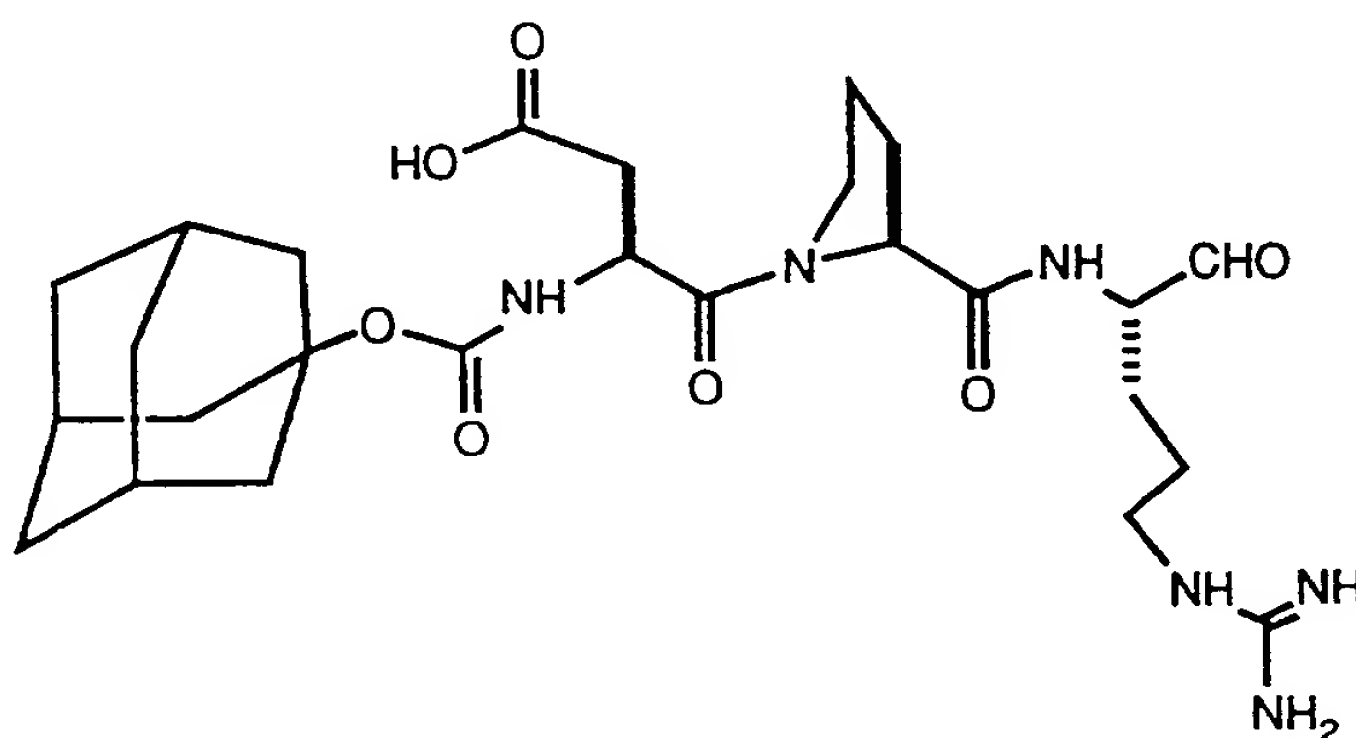
Example 10:Preparation of N-isobutyloxycarbonyl-L-Asp-L-Pro-L-argininal



Peptide aldehyde 10 was synthesized and purified in the same manner as described in Example 8. Here, N-Boc-L-proline was first attached to resin 7 followed by N-isobutoxycarbonyl-L-aspartic acid-beta-benzyl ester (in the place of N-Boc-L-glutamic acid- $\gamma$ -benzyl ester). Again, treatment with 50% trifluoroacetic acid was omitted after the last coupling. Fast atom bombardment mass spectrometry gave observed molecular weight of 470 a.m.u.; calculated molecular weight was 470 a.m.u.

Example 11:

Preparation of N-adamantyloxycarbonyl-L-Asp-L-Pro-L-argininal



11

2.5 g (12.6 mmole) adamantyloxycarbonyl fluoride was added to a mixture of 2.2 g (10 mmole)  $\alpha$ -N-(t-butoxycarbonyl)-L-aspartic acid-beta-(benzyl ester) in 50 mL saturated  $\text{NaHCO}_3$  and 30 mL tetrahydrofuran. After stirring for 2 hours at room temperature, the reaction mixture was poured into 100 mL 1 N hydrochloric acid, and extracted with ethyl acetate. The combined extracts were washed with water, dried over  $\text{MgSO}_4$ , and concentrated to an oil. The oil was taken up in ether, then precipitated by addition of hexanes. The supernatant was decanted and the liquid concentrated in vacuum to give a white foam.

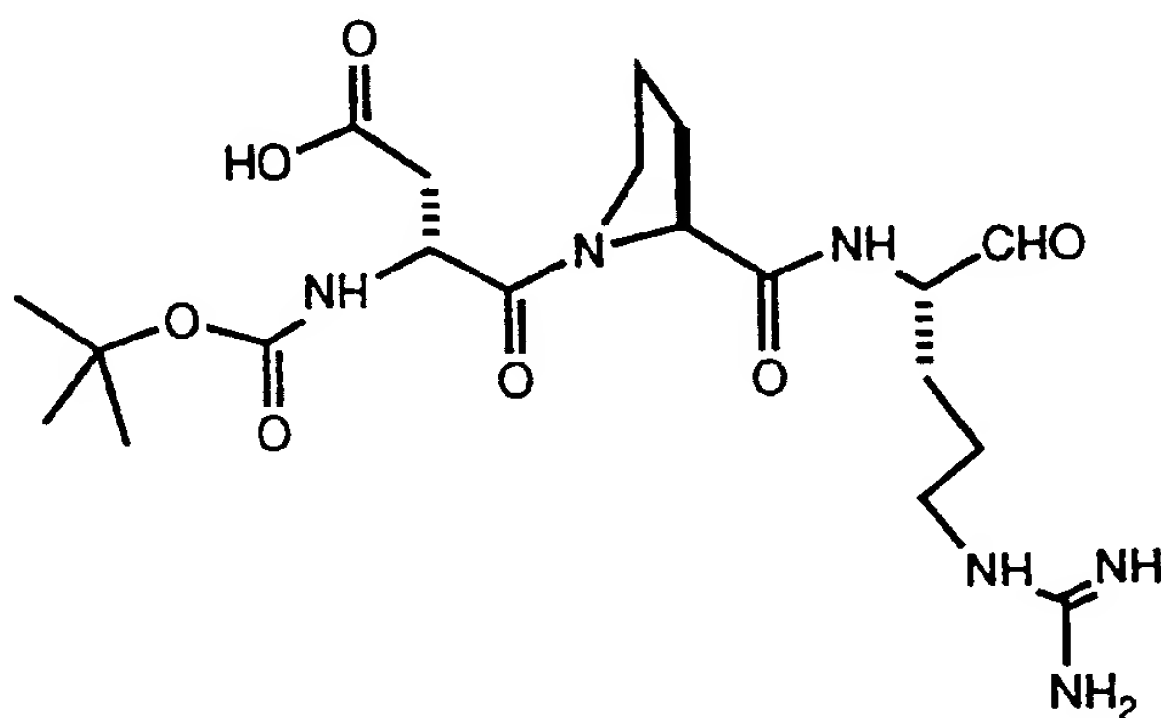
1.8 g (45% yield) N-adamantyloxycarbonyl-L-aspartic acid-gamma-benzyl ester was recovered.

Peptide aldehyde 11 was synthesized and purified in the same manner as described in Example 8.

5 Here, N-Boc-L-proline was first attached to resin 7 followed by N-adamantyloxycarbonyl-L-aspartic acid-gamma-benzyl ester (in the place of N-Boc-L-glutamic acid- $\gamma$ -benzyl ester). Again, treatment with 50% trifluoroacetic acid was omitted after the last coupling. Fast atom  
10 bombardment mass spectrometry gave observed molecular weight of 548 a.m.u.; calculated molecular weight was 548 a.m.u.

Example 12:

15 Preparation of N-t-butyloxycarbonyl-D-Asp-L-Pro-L-argininal



12

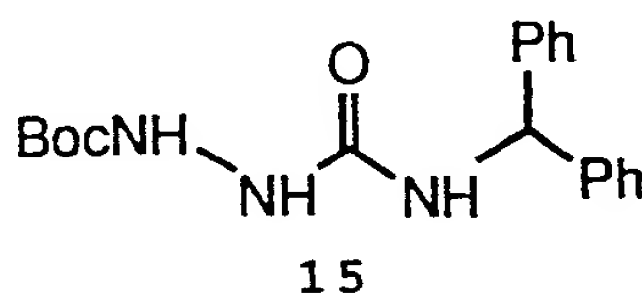
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Peptide aldehyde 12 was synthesized and purified in the same manner as described in Example 8. Here, N-Boc-L-proline was first attached to resin 7 followed by N-Boc-D-aspartic acid-beta-benzyl ester (in the place of N-Boc-L-glutamic acid- $\gamma$ -benzyl ester). Again, treatment with  
25 50% trifluoroacetic acid was omitted after the last coupling. Fast atom bombardment mass spectrometry gave observed molecular weight of 470 a.m.u.; calculated molecular weight was 470 a.m.u.

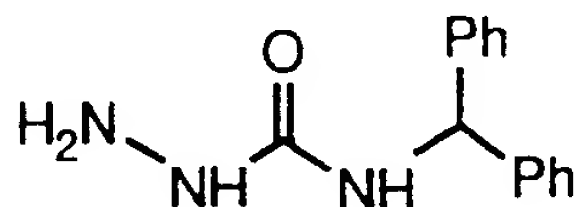
In the following examples the  $^1\text{H}$  NMR is consistent with the desired product in every case. The following examples are illustrated in Figs. 2 and 3.

5 Example 13:

Preparation of 1-t-butoxycarbonyl-semicarbazidyl-4-diphenylmethane

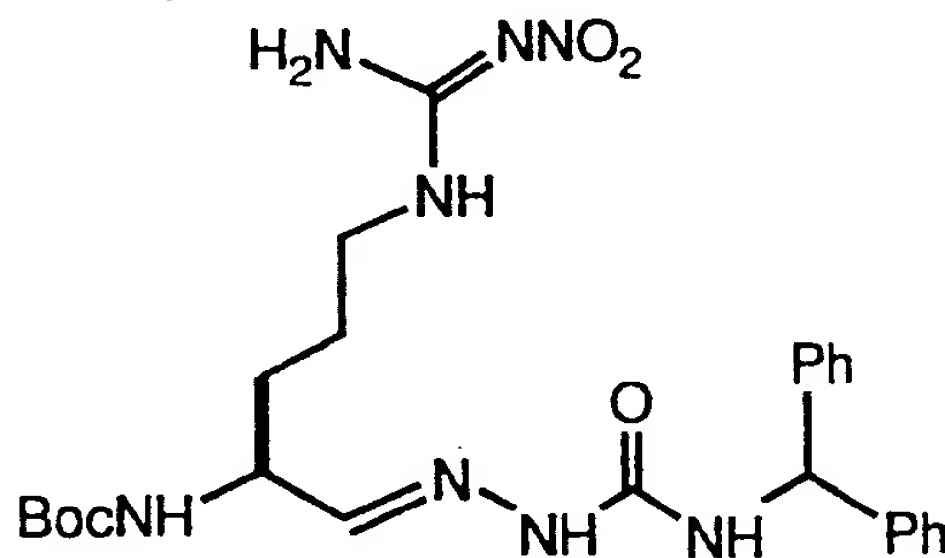


A solution of 16.2 g (0.10 mole) carbonyldiimidazole (CDI) in 225 mL dimethylformamide (DMF) was prepared at room temperature and allowed to stir under nitrogen. A solution of 13.2 g (0.100 moles) t-butyl carbazate (13) in 225 mL DMF was then added dropwise over a 30 min. period. Next a solution of 18.3 g (0.10 moles) diphenylmethylaniline 14 in 100 mL DMF was added over a 30 min. period. The reaction was allowed to stir at room temperature under nitrogen for one hour. Water (10 mL) was added and the mixture concentrated to about 150 mL under vacuum. This solution was poured into 500 mL water and extracted with 400 mL ethyl acetate. The ethyl acetate phase was extracted two times each with 75 mL 1N HCl, H<sub>2</sub>O, saturated NaHCO<sub>3</sub>, and brine, and dried with MgSO<sub>4</sub>. The mixture was filtered and the solution was concentrated to give 29.5 g (85% yield) of a white foam. This material could be purified by recrystallization from ethyl acetate/hexane, but was pure enough to use directly in the next step: mp 142-143°C. Anal. Calcd. for C<sub>19</sub>H<sub>23</sub>N<sub>3</sub>O<sub>3</sub>: C, 66.84; H, 6.79; N, 12.31. Found: C, 66.46; H, 6.75; N, 12.90.

Example 14:Preparation of semicarbazidyl-4-diphenylmethane  
trifluoroacetate salt

16

5  
A solution of 3.43 g (10 mmole) compound 15 in 12.5 mL dichloromethane was treated with 12.5 mL of  
10 trifluoroacetic acid (TFA) at 0°C and allowed to stir for 30 min at this temperature. After this time the solution was added dropwise to 75 mL ether. A precipitate formed, and the mixture was filtered and washed with ether. Weight of crude product was 2.7 g (80% yield): mp 182-  
15 184°C.

Example 15:Preparation of  $\alpha$ -N-(t-butoxycarbonyl)-N<sup>9</sup>-nitro-argininal-  
semicarbazonyl-4-N-diphenylmethane

17

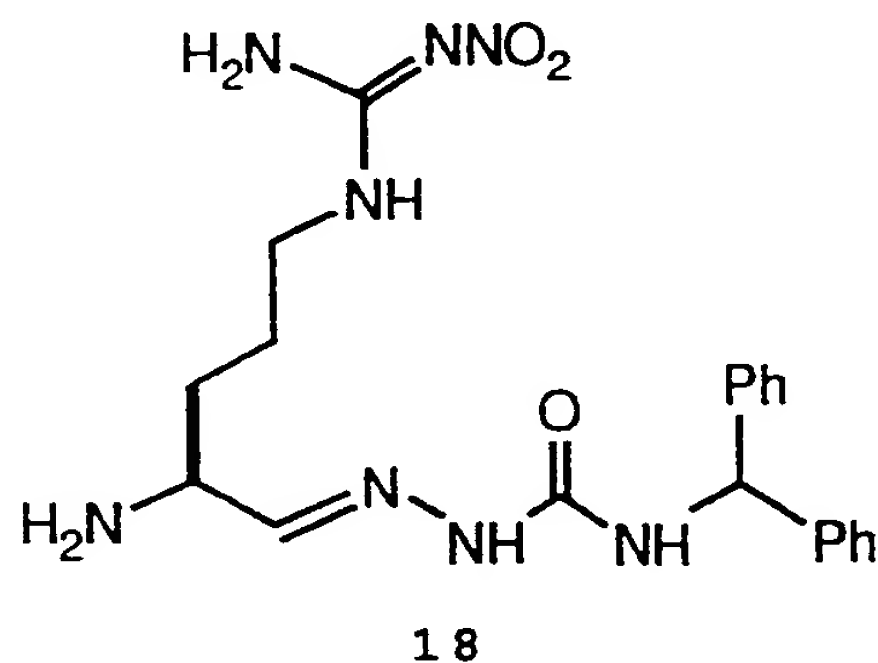
20  
A solution of 2.65 g (7.8 mmole) compound 16, and  
25 2.36 g (7.8 mmole) of 1 (alpha-N-(t-butoxycarbonyl)-N<sup>9</sup>-nitro-argininal) in 20 mL ethanol containing 6 mL of water, was treated with 1.2 g (8.8 moles) of sodium acetate and refluxed for one hour. This solution was allowed to cool and then poured into water and extracted  
30 three times with ethyl acetate. The combined organic

phase was washed with water, 0.1 N HCl, and brine, dried with MgSO<sub>4</sub>, and concentrated to a small volume. The white solid residue was recrystallized from acetonitrile/ether. This gave 3.2 g (78% yield base on 16): mp 78-79°C.

5

Example 16:

Preparation of N<sup>q</sup>-nitro-argininal-semicarbazonyl-4-N-diphenylmethane trifluoroacetate salt



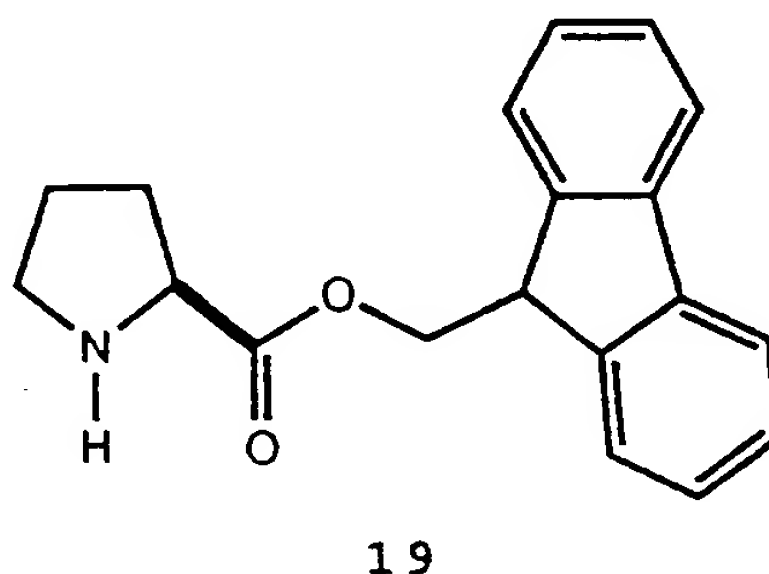
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A solution of 0.53 g (1.0 mmole) compound 17 in 5 mL dichloromethane was treated with 5 mL trifluoroacetic acid (TFA) at 0°C and allowed to stir for 30 minutes at this temperature. After this time the solution was added dropwise to 40 mL ether. A precipitate formed, and the mixture was filtered and washed with ether. This gave 0.51 g of a pure white solid (97% yield): mp 159-160°C.

20

Example 17:

Preparation of L-proline-9-fluorenemethyl ester p-toluenesulfonic acid salt

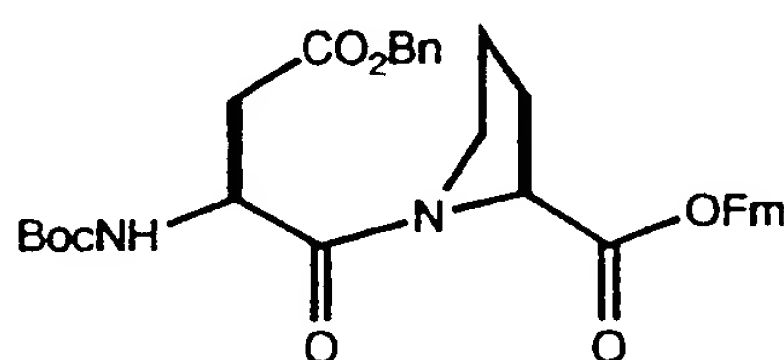


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A solution of L-proline 15.99 g (139.0 mmole), 9-fluorenemethanol 30.0 g (152.9 mmole), and p-toluenesulfonic acid in 600 mL of toluene was refluxed and water was removed with a Dean-Stark trap. After 26 hours, the reaction was concentrated to give 64 g (99% crude yield) of an oil which was used directly in the next step.

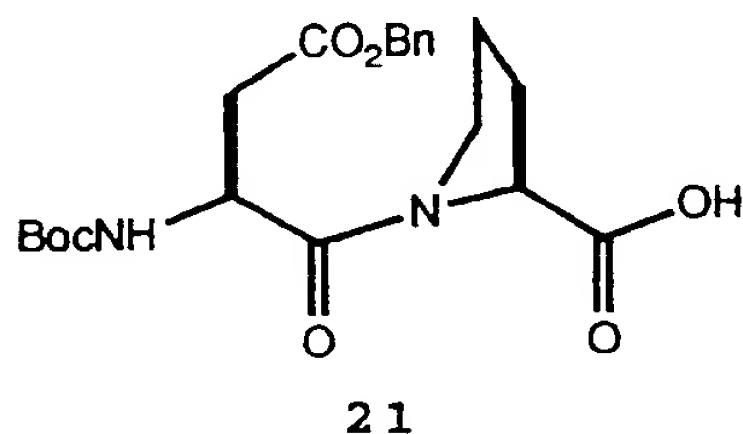
Example 18:

Preparation of  $\alpha$ -N-(t-butoxycarbonyl)-L-aspartyl-beta-(benzyl ester)-L-proline-9-fluorenemethyl ester



20

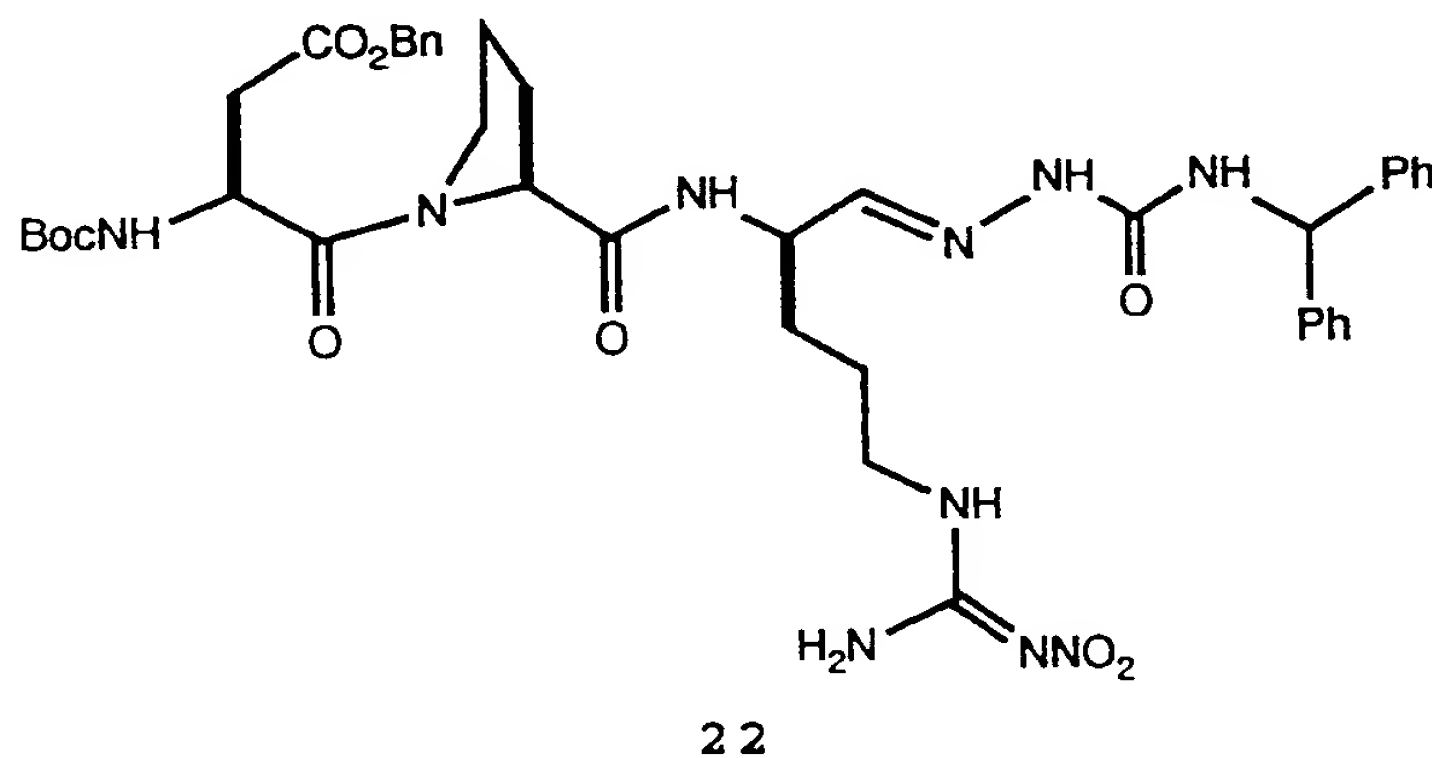
A solution of L-proline-9-fluorenemethyl ester p-toluenesulfonic acid salt **19** (15.44 g, 33.2 mmole),  $\alpha$ -N-(t-butoxycarbonyl)-L-aspartic acid-beta-(benzyl ester) (9.35 g, 41.9 mmole), benzotriazol-1-yloxy-tris-(dimethylamino)-phosponium-hexafluorophosphate (BOP reagent) 18.6 g (42.0 mmole) in 100 mL DMF was allowed to stir in an ice-bath. This solution was treated with 1-hydroxybenzotriazole hydrate (0.45 g, 3.34 mmole), diisopropylethylamine (19.0 mL, 198 mmole) and the reaction allowed to stir at 0-5°C for 1.5 hours. After this time the reaction mix was poured into 600 mL of ethyl acetate and extracted successively with saturated aqueous citric acid, water, saturated sodium bicarbonate, and finally brine. The organic phase was dried with MgSO<sub>4</sub> and concentrated under vacuum to give 18 g (91% crude yield) of an oil, which was used directly in the next step.

Example 19:Preparation of  $\alpha$ -N-(t-butoxycarbonyl)-L-aspartyl-beta-(benzyl ester)-L-proline

The crude oil from above,  $\alpha$ -N-(t-butoxycarbonyl)-L-aspartyl-beta-(benzyl ester)-L-proline 9-fluorenemethyl ester 20 (17.5 g, 29.2 mmole) was suspended in 250 mL triethylamine and allowed to reflux for 1 hour. This mixture was concentrated to an oil, dissolved in 600 mL of ethyl acetate. The ethyl acetate phase was washed once with citric acid, once with brine, dried with  $\text{MgSO}_4$ , and concentrated to give an oil. This material was purified by column chromatography (silica gel, 10-20% THF/DCM) to give 7.5 g (38% overall from 19).

Example 20:

Preparation of  $\alpha$ -N-(t-butoxycarbonyl)-L-aspartyl-beta-(benzyl ester)-L-prolyl-L-N<sup>9</sup>-nitro-argininal-semicarbazonyl-4-N-diphenylmethane

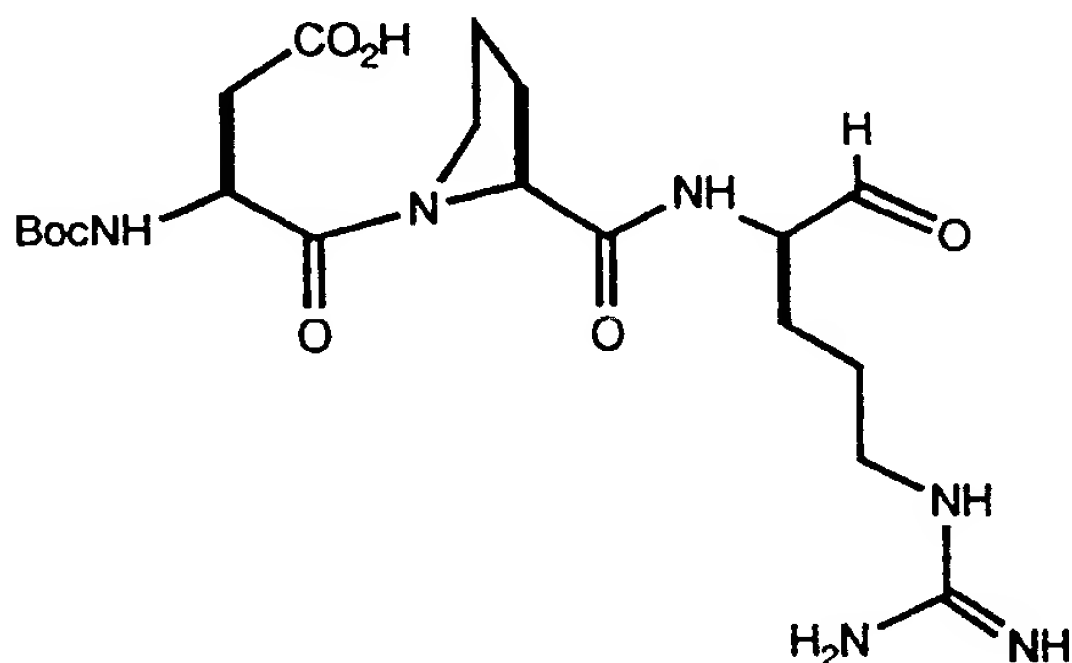


$\alpha$ -N-(t-butoxycarbonyl)-L-aspartyl-beta-(benzyl ester)-L-proline 21 (11.29 g, 26.9 mmole) was dissolved



in 60mL DMF. This solution was treated with N-methylmorpholine (NMM, 11.9 mL, 108 mmole), BOP (11.9 g, 27 mmole) and 18 (14.64 g, 28 mmole), then allowed to stir for 2h. This mixture was poured into 700 mL ethyl acetate and washed with 1N citric acid, saturated NaHCO<sub>3</sub>, water, and brine, dried with MgSO<sub>4</sub>, and concentrated to give a foam. This material was purified by column chromatography (silica gel, 6-20% IPA/DCM) to give 12.5 g (38% overall from 21).

10

Example 21:Preparation of  $\alpha$ -N-(t-butoxycarbonyl)-L-aspartyl-L-prolyl-L-argininal

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23

A solution of 22 (4.4 g, 5.1 mmole) in 85 mL methanol was treated with 20 mL water, 10.5 mL glacial acetic acid, 44 mL 1N HCl, and 2.2 g 10% Pd on carbon. This was hydrogenated at 11 psi with shaking for 70 min. The mixture was filtered and concentrated to a small volume. The resulting peptide aldehyde was then purified using reverse phase HPLC on a 10 micron particle size, 300 angstrom pore size C-18 column, eluting with a water-acetonitrile (both containing 0.01% trifluoroacetic acid) gradient, where the gradient ran from 5% to 40% acetonitrile. The column fractions were analyzed by analytical HPLC and fractions containing pure product were pooled and lyophilized. This gave 400 mg of pure 23, which was identical to the product from Example 9, along

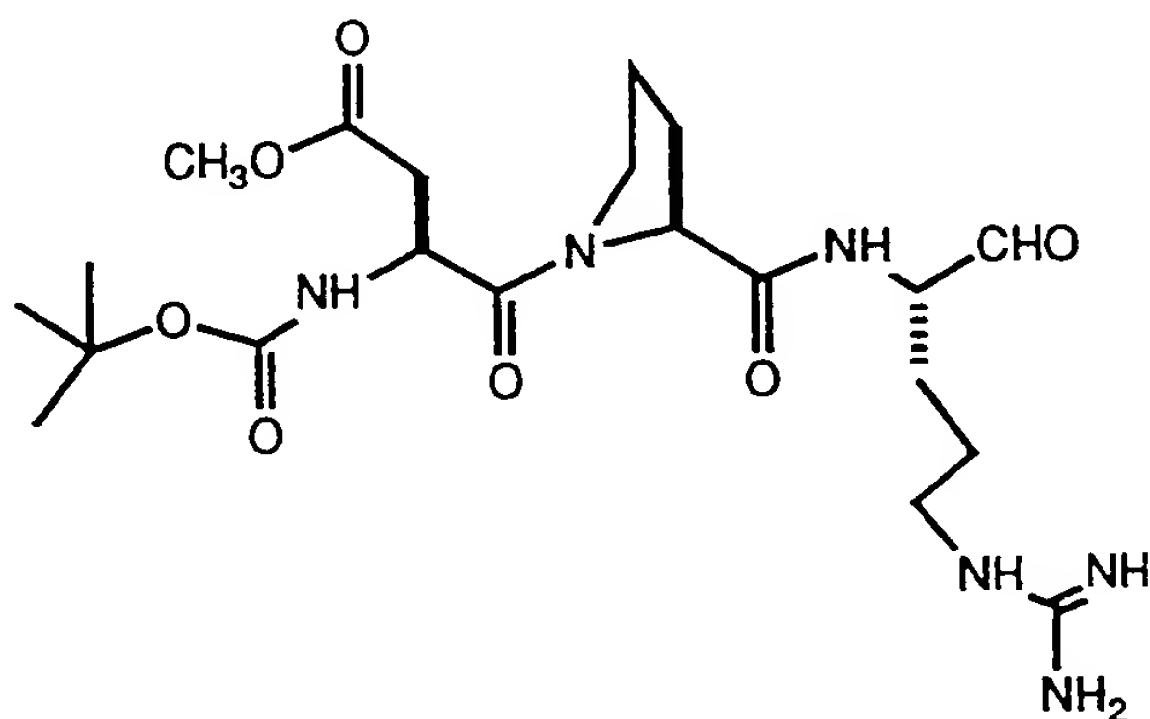
25

30

with 1.2 g of debenzylated starting material. The yield was 25% based on consumed starting material.

Example 22:

5 Preparation of N-t-butyloxycarbonyl-L-( $\beta$ -methyl ester)-  
Asp-L-Pro-L-argininal



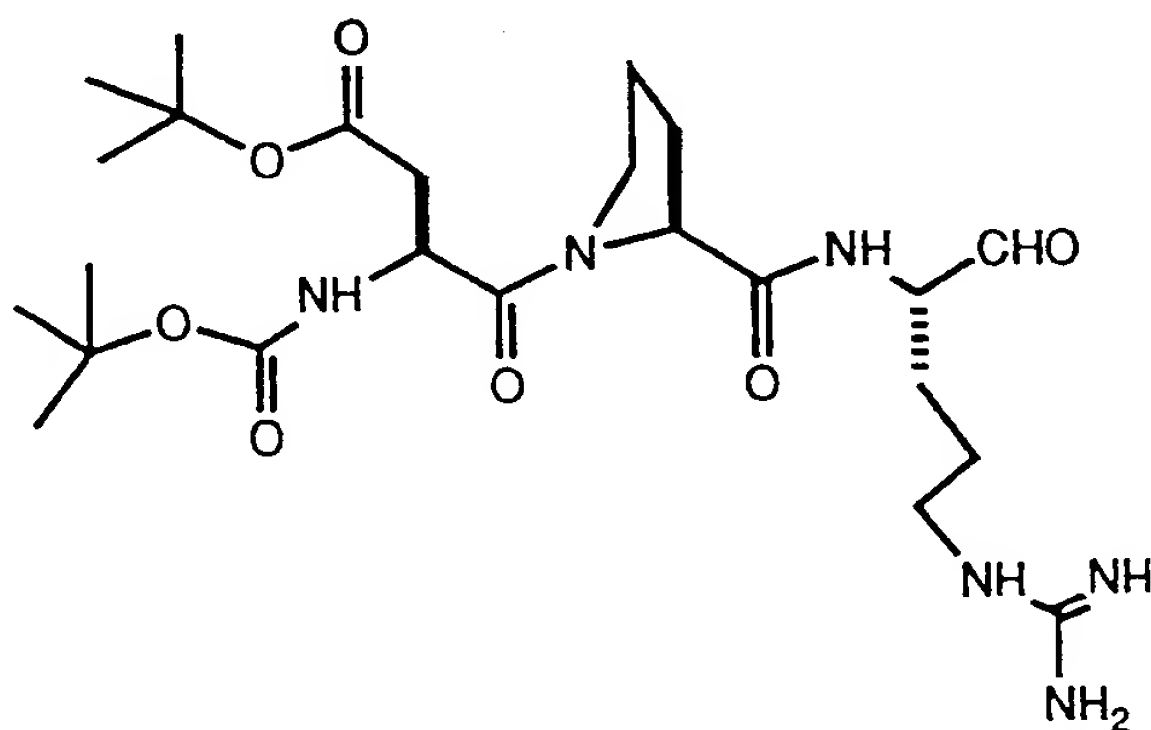
24

10

Peptide aldehyde **24** is synthesized and purified in the same manner as described in Example 8. Here, N-Boc-L-proline is first attached to resin **7** followed by N-Boc-L-( $\beta$ -methyl ester) aspartic acid in the place of N-Boc-L-glutamic acid- $\gamma$ -benzyl ester. Again, treatment with 50% trifluoroacetic acid is omitted after the last coupling.

Example 23:

Preparation of N-t-butyloxycarbonyl-L-( $\beta$ -t-butyl ester)-  
20 Asp-L-Pro-L-argininal

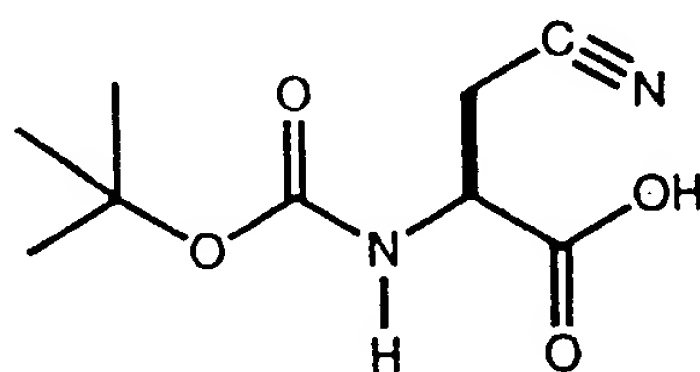


25

Peptide aldehyde 25 is synthesized and purified in the same manner as described in Example 8. Here, N-Boc-L-proline is first attached to resin 7 followed by N-Boc-L-( $\beta$ -t-butyl ester) aspartic acid in the place of N-Boc-L-glutamic acid- $\gamma$ -benzyl ester. Again, treatment with 50% trifluoroacetic acid is omitted after the last coupling.

Example 24:

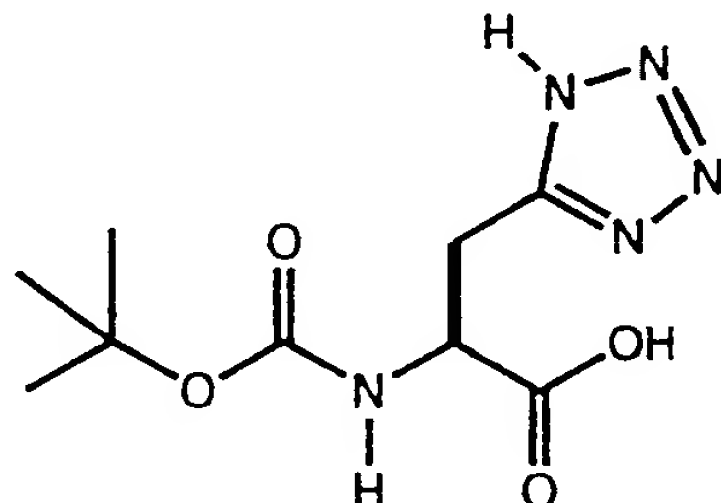
10 Preparation of 3-Cyano-2-(1,1-dimethylethoxy)  
methanamidopropionic acid



15

26

20.0 g (86 mmol, 1 equiv.) of Boc-L-Asparagine (from Bachem or Sigma) was dissolved in 120 mL of dry pyridine and 20.0 g (97 mmol, 1.3 equiv.) of dicyclohexylcarbodiimide dissolved in 60 mL of dry pyridine was added dropwise over a period of 30 minutes. The reaction was stirred for 3 hours at 23°C and filtered through a 2 $\mu$ m nylon filter. The filtrate was concentrated in vacuo on a rotary evaporator and 100 ml of water was added. The pH was adjusted to 10 with 40% sodium hydroxide (aq.) and the solution filtered through a 2 $\mu$ m nylon filter once again. The filtrate was passed through a 120 mL bed of Dowex 50X8-400 ion exchange resin and the resin washed with four column volumes of 1:1 methanol:water. The filtrate was concentrated in vacuo to yield 17.5 g (95% yield) of product as a white solid. <sup>1</sup>H-NMR (CD<sub>3</sub>OD): 4.40 p.p.m (m, 1H); 2.95 p.p.m. (m, 2H); 1.40 p.p.m. (s, 9H).

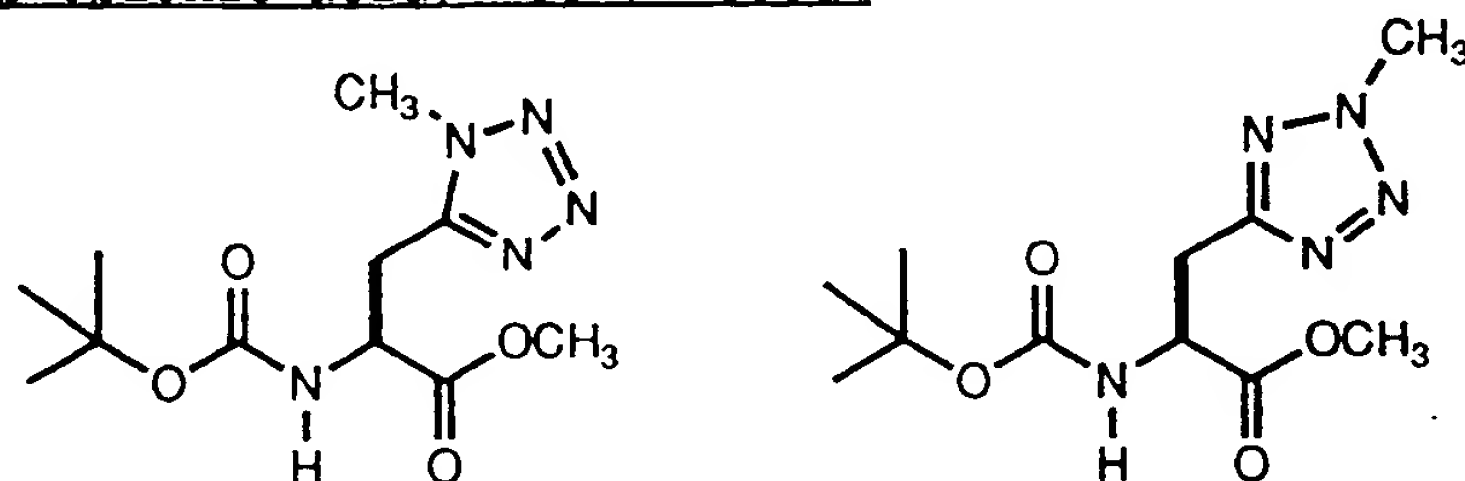
Example 25:Preparation of 3-Tetrazolyl-2-(1,1-dimethylethoxy)  
methanamidopropionic acid

27

17.5 g (82 mmol, 1 equiv.) of 3-cyano-2-(1,1-dimethylethoxy) methanamido-propionic acid 26 was dissolved in 125 mL of tetrahydrofuran and 40.5 g (129 mmol, 1.5 equiv.) tributyltin azide was added. The reaction mixture was brought to reflux and held there for 3 days. The reaction mixture was cooled and the volatiles removed in vacuo on a rotary evaporator. The residue was dissolved in 300 mL of 0.5 M sodium hydroxide and this aqueous solution was washed with ethyl acetate (4 x 100 mL). The aqueous layer was passed through a 125 mL bed of Dowex 50X8-400 ion exchange resin and the resin washed with four column volumes of 1:1 methanol:water. The volatiles were removed in vacuo on the rotary evaporator to yield 17.9 g of the product as a white solid (85 % yield). <sup>1</sup>H-NMR (CD<sub>3</sub>OD): 4.55 p.p.m (m, 1H); 3.40 p.p.m. (m, 2H); 1.40 p.p.m. (s, 9H). This material is suitable for use in solid-phase peptide synthesis.

Example 26:

Preparation of 3-(N-2-methyl)tetrazolyl-2-(1,1-  
dimethylethoxy)methanamidopropionic acid, methyl ester  
and 3-(N-3-Methyl)tetrazolyl-2-(1,1-dimethylethoxy)methan-  
amidopropionic acid, methyl ester



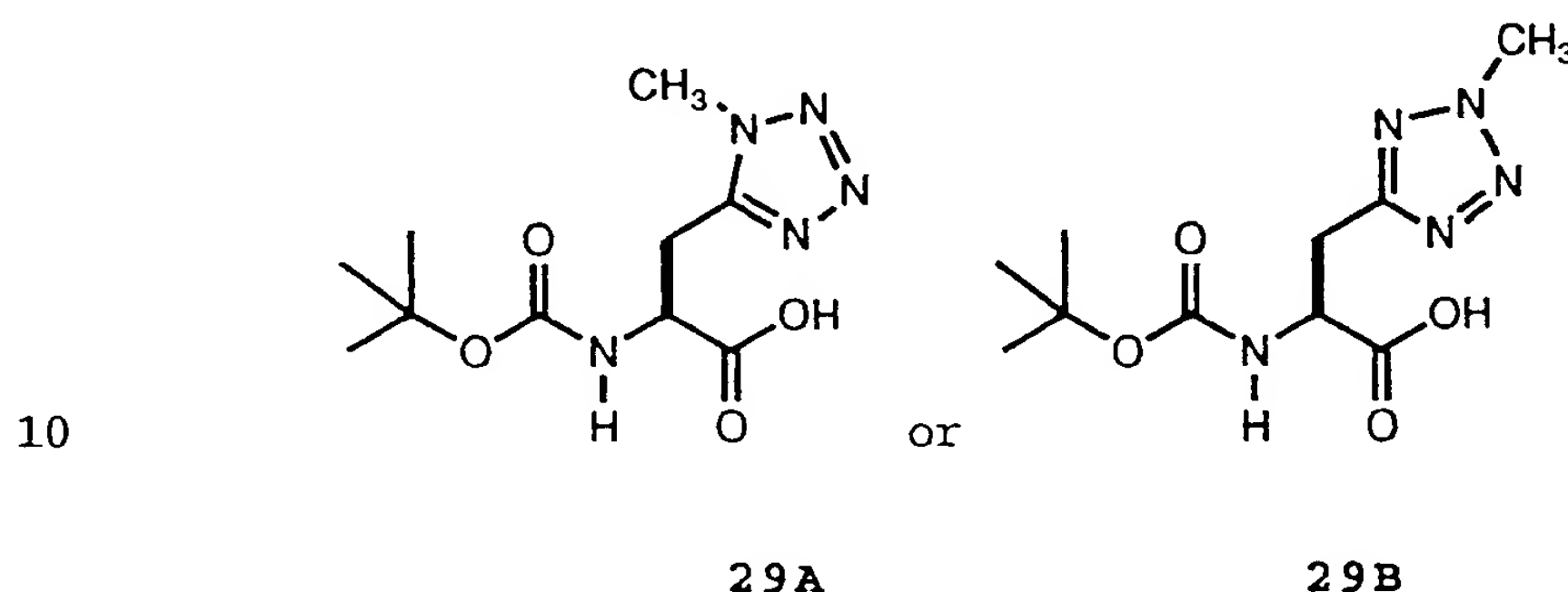
28

1.5 g (5.8 mmol, 1.0 equiv.) of 3-tetrazolyl-2-(1,1-  
 10 dimethylethoxy)methan-amidopropionic acid **27** was  
 dissolved in 13 mL of dry dimethylformamide and 3.9 g  
 (12.0 mmol, 2.1 equiv.) of cesium carbonate was added.  
 This was followed by the addition of 930  $\mu$ L (14.5 mmol,  
 2.5 equiv.) of methyl iodide via syringe. The reaction  
 15 mixture was stirred at 23°C for 3 hours and poured into 50  
 mL of 0.5M hydrochloric acid. The resulting mixture was  
 extracted with ethyl acetate (3 x 50 mL). The combined  
 organics were washed with 50 mL 0.5M hydrochloric acid, 50  
 mL saturated sodium bicarbonate, and 50 mL brine. After  
 20 drying over sodium sulfate, the organics were decanted and  
 the volatiles removed in vacuo on the rotary evaporator to  
 yield a mixture of the title compounds as a yellow oil.  
 The isomers were separated by chromatography on silica gel  
 (50% ethyl acetate/hexane) with one isomer eluting first  
 25 (R<sub>f</sub> = 0.3 vs. R<sub>f</sub> = 0.15 of the other isomer on silica gel  
 developing in 50% ethyl acetate/hexane). Fractions  
 containing pure product were combined and the volatiles  
 removed on the rotovap to yield 0.60 g of pure product for  
 each of the title compounds. <sup>1</sup>H-NMR (CDCl<sub>3</sub>): The second-  
 30 eluting isomer gave 5.8 p.p.m (d, 1H); 4.75 p.p.m (m, 1H);  
 4.05 p.p.m (s, 3H); 3.75 p.p.m. (s, 3H); 3.4 p.p.m (m,  
 2H); 1.5 p.p.m. (s, 9H). The first-eluting isomer gave:  
 5.75 p.p.m (d, 1H); 4.75 p.p.m (m, 1H); 4.30 p.p.m (s,

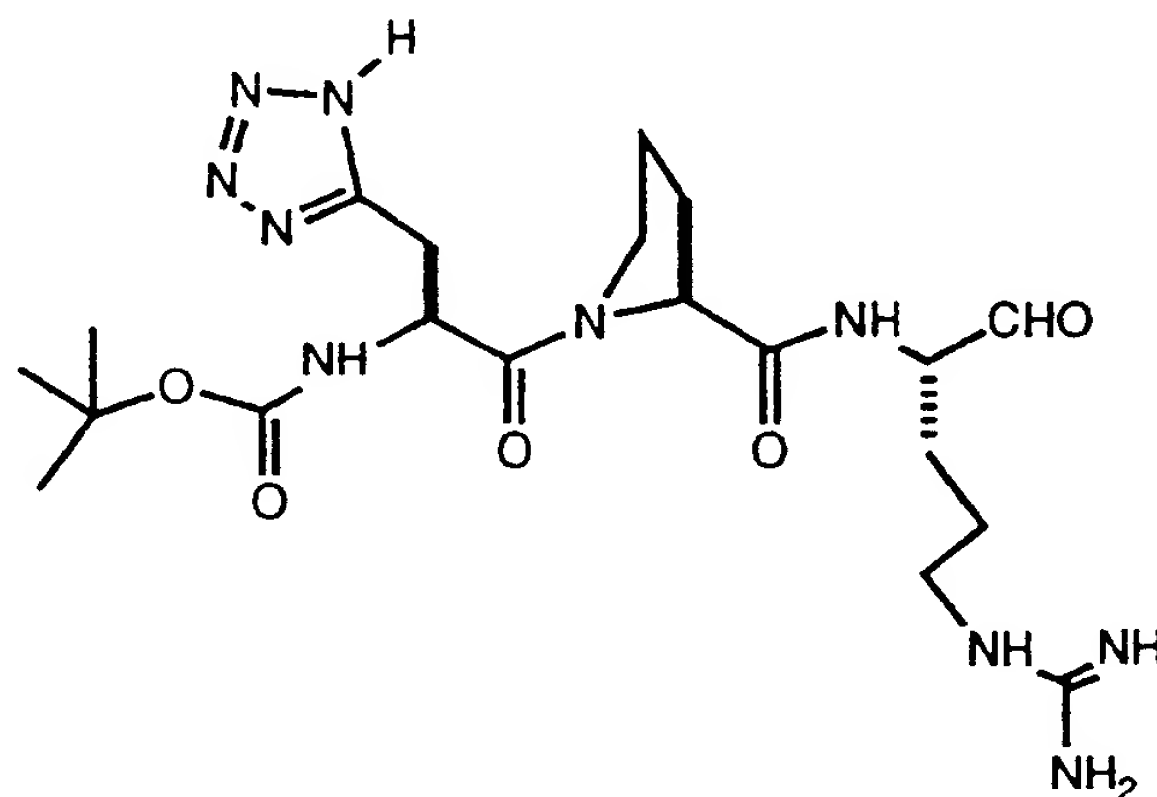
3H); 3.75 p.p.m. (s, 3H); 3.65 p.p.m (m, 2H); 1.7 p.p.m. (s, 9H).

Example 27:

- 5 Preparation of 3-(N-2-methyl)tetrazolyl-2-(1,1-  
dimethylethoxy)methanamidopropionic acid or 3-(N-3-  
methyl)tetrazolyl-2-(1,1-  
dimethylethoxy)methanamidopropionic



- 0.5 g (1.75 mmol, 1.0 equiv.) of 3-(N-2-
- 15 methyl)tetrazolyl-2-(1,1-dimethylethoxy)-  
methanamidopropionic acid methyl ester or 3-(N-3-  
methyl)tetrazolyl-2-(1,1-dimethylethoxy)-  
methanamidopropionic acid methyl ester is dissolved in 12  
mL of methanol and 2.3 mL (1.3 equiv.) of 1.0M lithium
- 20 hydroxide (aq.) is added. The reaction is stirred for 2  
hours at 23°C when starting material can no longer be seen  
by TLC analysis (1:1 ethyl acetate/hexane). The reaction  
mixture is passed through a 10 mL bed of Dowex 50X8-400  
ion exchange resin and the resin is washed with four
- 25 column volumes of 1:1 methanol:water. The solvents are  
removed in vacuo to yield the appropriate title product.

Example 28:Preparation of L-3-tetrazolyl-2-(1,1-dimethylethoxy)methanamidopropionyl-L-Pro-L-argininal

30

Peptide aldehyde 30 is synthesized and purified in the same manner as described in Example 8. Here, N-Boc-L-proline is first attached to resin 7 followed by 3-tetrazolyl-2-(1,1-dimethylethoxy)methanamidopropionic acid 27 (in the place of N-Boc-L-glutamic acid- $\gamma$ -benzyl ester). Again, treatment with 50% trifluoroacetic acid is omitted after the last coupling.

15

Example A:Potency - Determination of Inhibitor Constant,  $K_i$ 

The potency of peptide aldehydes, 8, 9, 11 and 12, as inhibitors of bovine and human pancreatic trypsin was quantified in vitro by determination of their inhibitor constants,  $K_i$ . Enzyme activity was determined using as substrate S-2222 [N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride where glutamyl side chain is 50% carboxylic acid and 50% methyl ester], purchased from Kabi Diagnostica. The substrate was made up in deionized water prior to use.

A 96-well microtiter plate was prepared for use in the assay by incubating each well with 300  $\mu$ L of 1% bovine serum albumin (in deionized water) for 30 minutes at 37°C, and washing three times with deionized water.

30

To each well was added 50  $\mu$ L TBS (0.1 M Tris, 0.14 M NaCl, pH 7.4), 50  $\mu$ L 2.5 nM trypsin and 50  $\mu$ L peptide aldehyde in TBS or TBS alone. After incubating for 15 minutes at 37°C, 50  $\mu$ L of S-2222 (at 37°C) at a specified concentration was added to each well. After mixing, the rate of substramte turnover at 37°C was measured for 30 minutes at 405nm (generation of p-nitroaniline). The initial S-2222 concentrations in the assay mixture were 0.45, 0.23, 0.11, 0.056, and 0.028 mM.

$K_i$  was determined graphically using a Dixon plot, as described in Dixon, Biochem. J. 55:170 (1953). Results are shown in Table 1 below.

Table 1.  $K_i$  For Peptide Aldehydes Against Trypsin.

Peptide Aldehyde	Structure	$K_i$ ( $\mu$ M)
N-t-butoxycarbonyl-L-Glu-L-Pro-L-Argininal	8	0.0014
N-t-butoxycarbonyl-L-Asp-L-Pro-L-Argininal	9	0.00045
N-adamantylloxycarbonyl-L-Asp-L-Pro-L-Argininal	11	0.0002
N-t-butoxycarbonyl-D-Asp-L-Pro-L-Argininal	12	0.045

Example B:

Specificity - Determination of IC<sub>50</sub>

The specificity of the peptide aldehydes (8 through 12) was determined in vitro by measurement of their IC<sub>50</sub> against other enzymes involved in hemostasis. A specific concentration of enzyme and its substrate were challenged with varying concentrations of inhibitor. IC<sub>50</sub> is that concentration of inhibitor giving 50% inhibition of substrate turnover, under the assay conditions. Specific assay procedures used are presented below. Tables 2a and 2b below show the results of the specificity assays.

">25" means less than 50% inhibition observed at highest concentration of inhibitor tested, 25  $\mu$ M inhibitor;

"<0.025" means greater than 50% inhibition observed at lowest concentration of inhibitor tested, 0.025  $\mu$ M;



"Inact." means no inhibition observed at highest concentration of inhibitor tested; and "ND" means not determined.

Table 2c and 2d shows % Selectivity relative to trypsin for each compound and commercial inhibitor tested. For each compound, % Selectivity is equal to  $[\text{IC}_{50} \text{ for Trypsin}) / \text{IC}_{50} \text{ for other enzyme}] \times 100$ . The lower the numerical value of % selectivity, the more selective the compound is as a trypsin inhibitor.

10

Table 2a.  $\text{IC}_{50}$  for Peptide Aldehydes and Commercial Inhibitors.

Compound		$\text{IC}_{50}$ ( $\mu\text{M}$ )				
Tested		Kallikrein	XIa	VIIa	Xa	Thrombin
15	8	6	4	>25	Inact.	14
	9	17	12	Inact.	>25	17
	10	8	13	Inact.	2	9
	11	2	6	>25	0.3	10
	12	>25	>25	Inact.	>25	Inact.
20	Aprotinin	0.2	2	>25	Inact.	Inact.
	Futhan	<0.025	<0.025	1.2	6.0	0.21
	FOY	1	1	>25	8	6
	Leupeptin	4	11	>25	25	20

25

Table 2b. IC<sub>50</sub> for Peptide Aldehydes and Commercial Inhibitor.

Compound		IC <sub>50</sub> (μM)				
	Tested	Protein C	Plasmin	tPA	UK	Trypsin
5	8	Inact.	0.6	Inact.	9	0.006
	9	Inact.	1.4	Inact.	25	0.014
	10	>25	1.2	>25	>25	<0.025
	11	>25	1.1	>25	19	<0.025
	12	>25	1.2	>25	17	0.084
10	Aprotinin	2	0.008	Inact.	ND	0.019
	Futhan	0.084	<0.025	0.37	ND	<0.025
	FOY	15	1.6	9	ND	0.25
	Leupeptin	>25	8	Inact.	Inact.	1.7

15

Table 2c. Calculated % Selectivity for Peptide Aldehydes and Commercial Inhibitors.

20	Compound	% Selectivity				
	Tested	Kallikrein	XIa	VIIa	Xa	Thrombin
25	8	0.10	0.15	<0.02	0	0.04
	9	0.08	0.12	0	<0.06	0.08
	10	<0.3	<0.19	0	<1.3	<0.28
	11	<1.3	<0.42	<0.1	<0.83	<0.25
	12	<0.34	<0.34	0	<0.34	0
	Aprotinin	9.5	0.95	<0.08	0	0
	Futhan	100	100	<2.1	<0.42	<0.12
30	FOY	25	25	<1.0	3.1	4.2
	Leupeptin	43	15	<6.8	6.8	8.5

Table 2d Calculated % Selectivity for Peptide Aldehydes and Commercial Inhibitors

5	Compound		% Selectivity			
	Tested	Protein C	Plasmin	tPA	UK	Trypsin
	8	0	1	0	0.07	100
	9	0	1	0	0.06	100
	10	<0.1	<2	<0.1	<0.1	100
10	11	<0.1	<2	<0.1	<0.1	100
	12	<0.3	7	<0.3	0.5	100
	Aprotinin	1	237	0	--	100
	Futhan	<30	100	<6.8	--	100
	FOY	1.7	16	2.7	--	100
15	Leupeptin	<6.8	21	0	0	100

(a) Preparation of Microtiter Plates.

96-well microtiter plates were prepared for use in these assays by incubating each well with 300  $\mu$ L 1% bovine serum albumin (in deionized water) for 30 minutes at 37°C, then washing three times with deionized water.

(b) Factor VIIa Assay.

Enzyme activity was determined using as substrate, S-2288 [D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide dihydrochloride], purchased from Kabi Diagnostica. The substrate was made up in deionized water prior to use.

Factor VIIa:rTF complex was prepared by adding to a polypropylene test tube 5 mL 25 nM recombinant human tissue factor (rTF), 2.5 mL 400 nM human Factor VIIa (in 0.4% bovine in TBS) and 2.5 mL 20 mM  $\text{CaCl}_2$ . Background control was prepared by adding to a second polypropylene test tube 1 mL 25 nM rTF, 0.5 mL 0.4% bovine serum albumin in TBS, and 0.5 mL 20 mM  $\text{CaCl}_2$ . Both solutions were then incubated for 30 minutes at room temperature before use in the assay.

The assay was run by combining in appropriate wells 50  $\mu$ L inhibitor in TBS or TBS alone and 100  $\mu$ L of Factor VIIa:rTF complex or background control, incubating this mixture for 30 minutes at room temperature, adding 50  $\mu$ L of 2 mM S-2288, incubating for an additional 30 to 60 minutes at room temperature, then reading the absorbance of the wells at 405 nm on a microtiter plate reader set with background subtraction at 650 nm.

10 (c) Factor Xa Assay.

Enzyme activity was determined using as substrate, Pefachrome Xa [N-methoxycarbonyl-D-cyclohexylalanyl-L-glycyl-L-arginine p-nitroanilide acetate], purchased from Centerchem, Inc. The substrate was made up in deionized water prior to use.

The assay was run by combining in appropriate wells 50  $\mu$ L of inhibitor in TBS or TBS alone and 50  $\mu$ L 30 nM human Factor Xa (or TBS as background control) and 50  $\mu$ L TBS, incubating this mixture for 30 minutes at room temperature, adding 50  $\mu$ L of 1 mM Pefachrome Xa, incubating for an additional 30 minutes at room temperature, then reading the absorbance of the wells at 405 nm on a microtiter plate reader set with background subtraction at 650 nm.

25

(d) Factor XIa Assay.

Enzyme activity was determined using as substrate, S-2366 [L-pyroglutamyl-L-prolyl-L-arginine-p-nitroanilide hydrochloride], purchased from Kabi Diagnostica. The substrate was made up in deionized water prior to use.

30

The assay was run by combining in appropriate wells 50  $\mu$ L of inhibitor in TBS or TBS alone and 50  $\mu$ L of 5 nM human Factor XIa (or TBS as background control) and 50  $\mu$ L TBS, incubating this mixture for 30 minutes at room temperature, adding 50  $\mu$ L of 2 mM S-2366, incubating for an additional 30 minutes at room temperature, then reading the absorbance of the wells at 405 nm on a microtiter plate reader set with background subtraction at 650 nm.

35

(e) Thrombin Assay.

Enzyme activity was determined using as substrate, S-2238 [D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride], purchased from Kabi Diagnostica. The substrate was made up in deionized water prior to use.

The assay was run by combining in appropriate wells 50  $\mu$ L TBS and 50  $\mu$ L inhibitor in TBS or TBS alone and 50  $\mu$ L 20 nM bovine thrombin (or TBS as background control), incubating this mixture for 30 minutes at room temperature, adding 50  $\mu$ L 1 mM S-2238, incubating for an additional 30 minutes at room temperature, then reading the absorbance of the wells at 405 nm on a microtiter plate reader set with background subtraction at 650 nm.

(f) Plasmin Assay.

Enzyme activity was determined using as substrate, S-2251 [D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride], purchased from Kabi Diagnostica. The substrate was made up in deionized water prior to use.

The assay was run by combining in appropriate wells 50  $\mu$ L of inhibitor in TBS or TBS alone and 50  $\mu$ L 25 nM human plasmin (or TBS as background control) and 50  $\mu$ L TBS, incubating this mixture for 30 minutes at room temperature, adding 50  $\mu$ L 2 mM S-2251, incubating for an additional 60 to 120 minutes at room temperature, then reading the absorbance of the wells at 405 nm on a microtiter plate reader set with background subtraction at 650 nm.

(g) Protein C Assay.

Enzyme activity was determined using as substrate, Pefachrome PC [ $\delta$ -carbobenzoxymethyl-L-lysyl-L-arginine-p-nitroanilide], purchased from Centerchem, Inc. The substrate was made up in deionized water prior to use. Protac used to activate Protein C was obtained from American Diagnostics.

Activated human Protein C was prepared by adding to a polypropylene test tube 5 mL of pooled human plasma diluted 1:8 with TBS and 5 mL of 0.117 Units/mL Protac, then incubating for 60 minutes at 37°C.

- 5        The assay was run by combining in appropriate wells 50 µL of inhibitor in TBS or TBS alone and 100 µL activated Protein C, incubating this mixture for 30 minutes at room temperature, adding 50 µL 2 mM Pefachrome PC, incubating for an additional 60 minutes at room  
10       temperature, then reading the absorbance of the wells at 405 nm on a microtiter plate reader set with background subtraction at 650 nm.

(h) Tissue Plasminogen Activator Assay.

- 15       Enzyme activity was determined using as substrate, S-2288, purchased from Kabi Diagnostica. The substrate was made up in deionized water prior to use.

- The assay was run by combining in appropriate wells 50 µL of inhibitor in TBS or TBS alone and 50 µL 50 nM  
20       recombinant tissue plasminogen activator (or TBS as background control) and 50 µL TBS, incubating this mixture for 30 minutes at room temperature, adding 50 µL 2 mM S-2288, incubating for an additional 60 to 120 minutes at room temperature, then reading the absorbance of the wells  
25       at 405 nm on a microtiter plate reader set with background subtraction at 650 nm.

(i) Trypsin Assay.

- Enzyme activity was determined using as substrate S-  
30       2222, purchased from Kabi Diagnostica. The substrate was made up in deionized water prior to use.

- The assay was run by combining in appropriate wells 50 µL TBS and 50 µL inhibitor in TBS or TBS alone and 50 µL of 40 nM bovine trypsin (or TBS as background control),  
35       incubating this mixture for 30 minutes at room temperature, adding 50 µL 1.8 mM S-2222, incubating for an additional 30 minutes at room temperature, then reading

the absorbance of the wells at 405 nm on a microtiter plate reader set with background subtraction at 650 nm.

(j) Kallikrein Assay.

5 Enzyme activity was determined using as substrate S-2302 [D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride], purchased from Kabi Diagnostica. The substrate was made up in deionized water prior to use.

The assay was run by combining in appropriate wells  
10 50  $\mu$ L TBS and 50  $\mu$ L of inhibitor in TBS or TBS alone and 50  $\mu$ L of 4 to 9 nM human kallekrein (or TBS as background control), incubating this mixture for 30 minutes at room temperature, adding 50  $\mu$ L of 1 mM S-2302, incubating for an additional 30 minutes at room temperature, then reading  
15 the absorbance of the wells at 405 nm on a microtiter plate reader set with background subtraction at 650 nm.

(k) Urokinase Assay.

Enzyme activity was determined using as substrate S-  
20 2444 [L-pyroglutamyl-L-glycyl-L-arginine-p-nitroanilide hydrochloride], purchased from Kabi Diagnostica. The substrate was made up in deionized water prior to use.

The assay was run by combining in appropriate wells  
50  $\mu$ L inhibitor in TBS or TBS alone and 50  $\mu$ L of 270 U/mL  
25 human urokinase (or TBS as background control) and 50  $\mu$ L TBS, incubating this mixture for 30 minutes at room temperature, adding 50  $\mu$ L 1 mM S-2444, incubating for an additional 30 minutes at room temperature, then reading  
the absorbance of the wells at 405 nm on a microtiter  
30 plate reader set with background subtraction at 650 nm.

Example C:

Animal Model for Pancreatitis

Niederau et al., Gastroenterology, 88:1192-1204  
35 (1985) showed that acute necrotizing pancreatitis can be induced in mice by intraperitoneal (IP) injections of caerulein. When so induced, serum amylase levels were

found to rise and fall with the severity and course of inflammatory process.

Pancreatitis was induced in fasting male balb/c mice weighing between 18 to 20 g by giving them three IP  
 5 injections of caerulein, with each dose at 100 µg/kg body weight. The injections of each were given at two hour intervals over a 6 hour period. The ability of N-Boc-L-Asp-Pro-Arg-al (compound 9) to inhibit the induced  
 10 pancreatitis was tested by injection into caerulein-treated mice. The inhibitor was dissolved into TBS, then injected IP into the mice. The first injection was given 1/4 hour prior to the caerulein treatment, then one hour after each caerulein injection. Inhibitor dose tested was 50 mg/kg body weight.

15 A blood serum sample was drawn and tested for amylase concentration. The blood sample was obtained by periorbital bleeding into heparized tubes 4 hours after the last injection of inhibitor. After centrifuging to remove the blood cells, the serum was the diluted 1:10 in  
 20 TBS and assayed with Sigma Diagnostics Amylase reagent. The kinetic change in absorbance was measured at 405nm for 1 minute, then was converted into U/ml amylase activity.

TABLE 3 (Amylase Activity (U/ml))

25		Mean $\pm$ S.D	n
	Saline + caerulein	64.4 $\pm$ 14.4a	7
30	N-Boc-L-Asp-Pro-Arg-al + caerulein	40.8 $\pm$ 5.2a,b	8
	Control (no caerulein)	10.4 $\pm$ 1.6	5

a, -  $p < 0.01$  vs control by Newman-Keuls test

35 b, -  $p < 0.01$  vs saline + caerulein by Newman-Keuls test

These data show the protective effect of inhibitors of this invention in vivo.



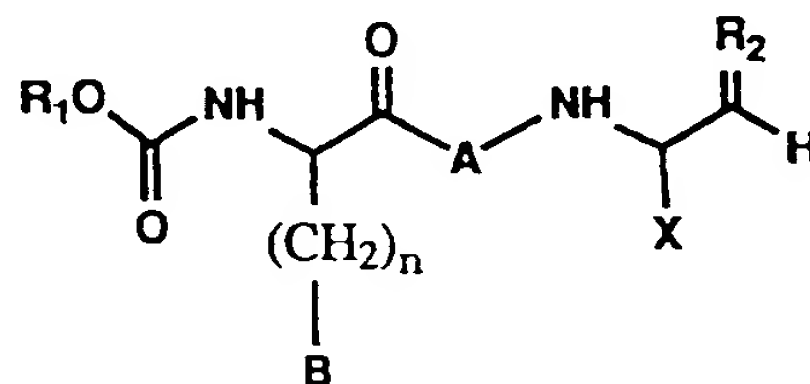
Having described the invention, it will be apparent to one of ordinary skill in the art that changes and modifications can be made without departing from the spirit and scope of the invention as set forth herein.

5 Other embodiments are within the following claims.

Claims

1. A compound of the formula:  

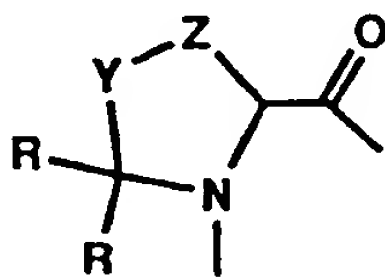
$$\text{Pr}-\text{A}_1-\text{A}_2-\text{A}_3$$
 wherein Pr is a hydrophobic group;  
 5      $\text{A}_1$  is selected from the group consisting of Glu, Asp, and an equivalent of Glu and Asp,  
 $\text{A}_2$  is Pro or an equivalent of Pro,  
 $\text{A}_3$  is Arg aldehyde and an equivalent of said Arg aldehyde, and  
 10     said compound is active to inhibit trypsin activity with an  $\text{IC}_{50}$  for trypsin at least 50 fold lower than the  $\text{IC}_{50}$  for kallikrein, protein C, plasmin, factors Xa, XIa, and VIIa, thrombin, tPA, and urokinase.
- 15     2. A compound of claim 1 wherein  $\text{A}_3$  is argininal.
3. A compound of claim 2 wherein  $\text{A}_1$  is Glu or Asp.
4. A compound of claim 2 wherein  $\text{A}_1$  is a  $\gamma$ -R' ester  
 20 of Glu, a  $\beta$ -R' ester of Asp or an R'-substituted tetrazole which replaces the  $\gamma$ -carboxy group of Glu or the  $\beta$ -carboxy group of Asp, wherein R' is hydrogen, lower alkyl of 1 to 6 carbon atoms or aralkyl of about 6 to about 15 carbon atoms.
- 25     5. The compound of claim 1 wherein said protecting group is butoxycarbonyl or adamanyloxy-carbonyl or an equivalent thereof.
- 30     6. A compound of the formula:



wherein  $R_1$  is selected from the group consisting of a branched alkyl of between 4 and 10 carbon atoms, cyclic or polycyclic alkyl of between 4 to 10 carbon atoms wherein said cyclic alkyl or polycyclic alkyl may be substituted with one or more alkyl groups of 1 to 5 carbon atoms;

$n$  is 1, 2 or 3;

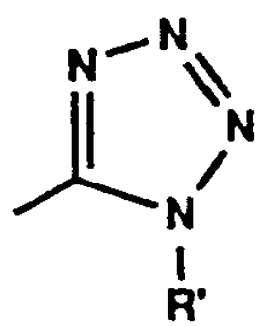
A is a group having the formula:



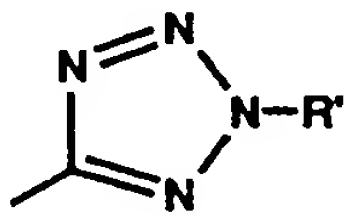
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wherein Y and Z are independently selected from a direct link, an oxygen atom and a methylene group(s), where only one of Y and Z can be a direct link or an oxygen atom and each R is independently H or an alkyl group of 1 or 2 carbon atoms;

B is selected from a group consisting of  $-\text{CO}_2\text{H}$ ,  $-\text{CO}_2\text{R}'$ ,



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, and wherein  $R'$  is H, lower alkyl of 1 to 6 carbons, or aralkyl of about 6 to about 15 carbon atoms;

X is selected from the group consisting of  $-(\text{CH}_2)_3-\text{NH}-\text{C}(=\text{NH})-\text{NH}_2$ ,  $-(\text{CH}_2)_3-\text{C}(=\text{NH})-\text{NH}_2$ , 4-amidinophenylmethyl, 4-guanidinylphenylmethyl, 4-aminomethylphenylmethyl and mono- and di-substituted N-alkyl derivatives thereof, wherein said N-alkyl is selected from the group consisting of methyl, ethyl, propyl, isopropyl, butyl and isobutyl; and

$R_2$  is selected from the group consisting of oxygen, and  $-\text{N}-\text{NR}_3-\text{C}(=\text{O})-\text{NH}_2$ , wherein  $R_3$  is selected from the group consisting of hydrogen, an alkyl group of between 1 and 6 carbon atoms, a phenyl group, or aralkyl group of

30

between 7 and 9 carbon atoms and a peptide or peptide analog wherein said  $R_2$  is hydrolyzed to give a derivative with an oxygen atom at a pH below 6.0; and pharmaceutically acceptable salts thereof.

5

5. A compound of claim 4 wherein  $R_1$  is selected from the group consisting of isobutyl, t-butyl and adamantyl.

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6. A compound of claim 5 wherein  $R_2$  is oxygen.

7. A compound of claim 6 wherein A is L-proline.

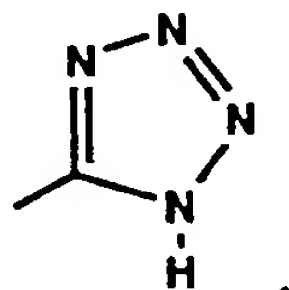
8. A compound of claim 7 wherein X is  
15  $-(CH_2)_3-NH-C(=NH)-NH_2$  and n is 1 or 2.

9. A compound of claim 8 wherein B is  $-CO_2H$ .

10. A compound of claim 8 wherein B is  $-CO_2CH_3$ .

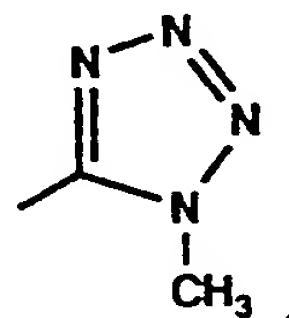
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11. A compound of claim 8 wherein B is



12. A compound of claim 8 wherein B is

25



13. A compound of claim 7 wherein X is  
30  $-(CH_2)_3-C(=NH)-NH_2$  and n is 1 or 2.

30

14. A compound of claim 7 wherein X is 4-amidinophenylmethyl and n is 1 or 2.

15. A compound of claim 7 wherein X is 4-guanidinylphenylmethyl and n is 1 or 2.

5 16. A compound of claim 7 wherein X is 4-aminomethylphenylmethyl and n is 1 or 2.

17. A compound of claim 5 wherein R<sub>2</sub> is -N-NH-C(=O)-NH<sub>2</sub>.

10

18. A compound of claim 17 wherein A is L-proline.

19. A compound of claim 18 wherein X is -(CH<sub>2</sub>)<sub>3</sub>-NH-C(=NH)-NH<sub>2</sub> and n is 1 or 2.

15

20. A compound of claim 18 wherein X is -(CH<sub>2</sub>)<sub>3</sub>-C(=NH)-NH<sub>2</sub> and n is 1 or 2.

21. A compound of claim 18 wherein X is 4-amidinophenylmethyl and n is 1 or 2.

20

22. A compound of claim 18 wherein X is 4-guanidinylphenylmethyl and n is 1 or 2.

23. A compound of claim 18 wherein X is 4-aminomethyl-phenylalanine and n is 1 or 2.

25

24. A compound of claim 4 wherein A is selected from the group consisting of azetidine carboxylic acid, L-proline, β-methyl-L-proline β,β-dimethyl-L-proline and 3,4-dehydro-L-proline.

30

25. A pharmaceutical composition for prevention or treatment of a condition characterized by an elevated trypsin activity in a mammal, comprising a pharmaceutically acceptable carrier, and a pharmaceutically effective amount of compound of claim 1 or 4.

35

26. A pharmaceutical composition for prevention or treatment of a condition characterized by an elevated trypsin activity in a mammal, comprising a  
5 pharmaceutically acceptable carrier, and a pharmaceutically effective amount of compound of claim 5.

27. A pharmaceutical composition for prevention or treatment of a condition characterized by an elevated  
10 trypsin activity in a mammal, comprising a pharmaceutically acceptable carrier, and a pharmaceutically effective amount of compound of claim 6 or 17.

15 28. A pharmaceutical composition for prevention or treatment of a condition characterized by an elevated trypsin activity in a mammal, comprising a pharmaceutically acceptable carrier, and a pharmaceutically effective amount of compound of claim 7  
20 or 18.

29. A pharmaceutical composition for prevention or treatment of a condition characterized by an elevated trypsin activity in a mammal, comprising a  
25 pharmaceutically acceptable carrier, and a pharmaceutically effective amount of compound of claim 8, 13, 14, 15 or 16.

30 30. A pharmaceutical composition for prevention or treatment of a condition characterized by an elevated trypsin activity in a mammal, comprising a pharmaceutically acceptable carrier, and a pharmaceutically effective amount of compound of claim 9,  
35 10, 11 or 12.

31. A pharmaceutical composition for prevention or treatment of a condition characterized by an elevated trypsin activity in a mammal, comprising a

pharmaceutically acceptable carrier, and a pharmaceutically effective amount of compound of claim 19, 20, 21, 22 or 23.

5           32. A method for preventing or treating a condition characterized by an elevated trypsin activity in a mammal comprising administering to said mammal a pharmaceutically effective dose of the composition of claim 24.

10           33. A method for preventing or treating a condition characterized by an elevated trypsin activity in a mammal, comprising administering to said mammal a pharmaceutically effective dose of the composition of claim 25.

15           34. A method for preventing or treating a condition characterized by an elevated trypsin activity in a mammal, comprising administering to said mammal a pharmaceutically effective dose of the composition of claim 26.

20           35. A method for preventing or treating a condition characterized by an elevated trypsin activity in a mammal, comprising administering to said mammal a pharmaceutically effective dose of the composition of claim 27.

25           36. A method for preventing or treating a condition characterized by an elevated trypsin activity in a mammal, comprising administering to said mammal a pharmaceutically effective dose of the composition of claim 28.

30           37. A method for preventing or treating a condition characterized by an elevated trypsin activity in a mammal, comprising administering to said mammal a pharmaceutically effective dose of the composition of claim 29.

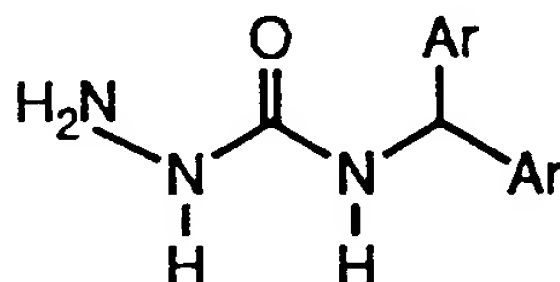
35           38. A method for preventing or treating a condition characterized by an elevated trypsin activity in a mammal, comprising administering to said mammal a pharmaceutically effective dose of the composition of claim 30.

39. A method for preventing or treating a condition characterized by an elevated trypsin activity in a mammal, comprising administering to said mammal a pharmaceutically effective dose of the composition of claim 31.

40. Method for synthesis of a peptide aldehyde, comprising the steps of:

reacting a semicarbazide having the formula:

10



wherein Ar is phenyl or an equivalent thereof,  
with an  $\alpha$ -N-protected amino aldehyde to produce a  
protected semi-carbazone,  
deprotecting the  $\alpha$ -amino group of said protected  
semi-carbazone, and  
reacting the deprotected semi-carbazone with an N-  
protected amino acid to produce an N-protected peptide  
aldehyde or analog thereof.

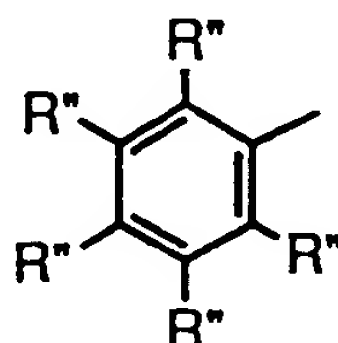
41. The method of claim 40, wherein said Ar is phenyl.

42. The method of claim 40 wherein said amino aldehyde is argininal.

43. The method of claim 40 wherein said deprotecting and reacting the deprotected semi-carbazone steps are repeated a plurality of times with either the same said N-protected amino acid or a different N-protected amino acid.

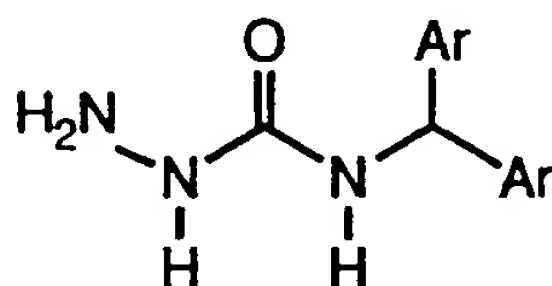
44. The method of claim 40 wherein said Ar has the formula:





wherein each R'' is selected independently from the group  
consisting of H, methyl, methoxy, halogen, ethyl and  
5 ethoxy.

45. Protected semicarbazone formed by reacting a  
semicarbazide having the formula:



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wherein Ar is phenyl or an equivalent thereof, with an  $\alpha$ -  
N-protected amino aldehyde to produce a protected semi-  
carbazone.

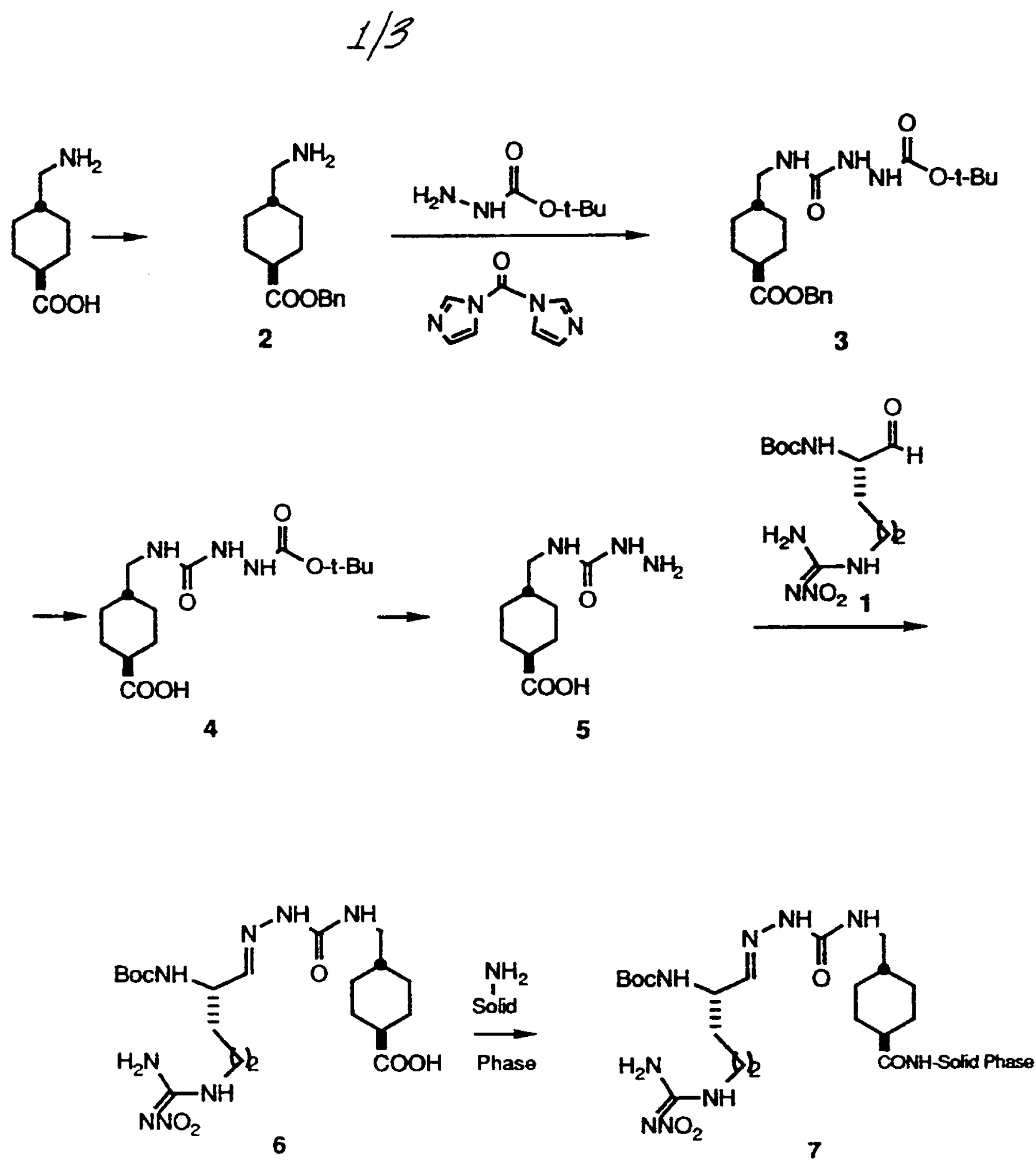


FIG. 1

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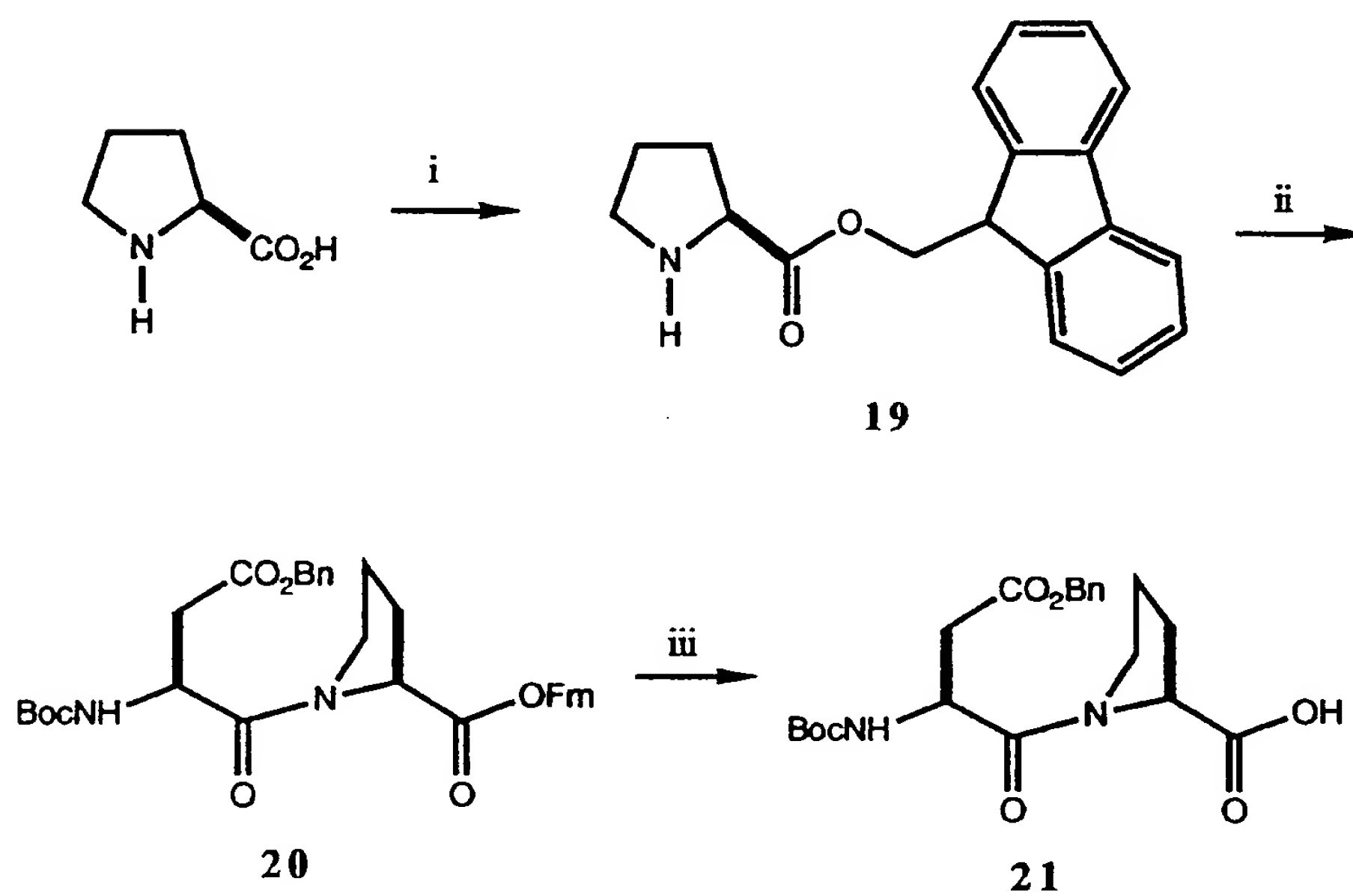


FIG. 2

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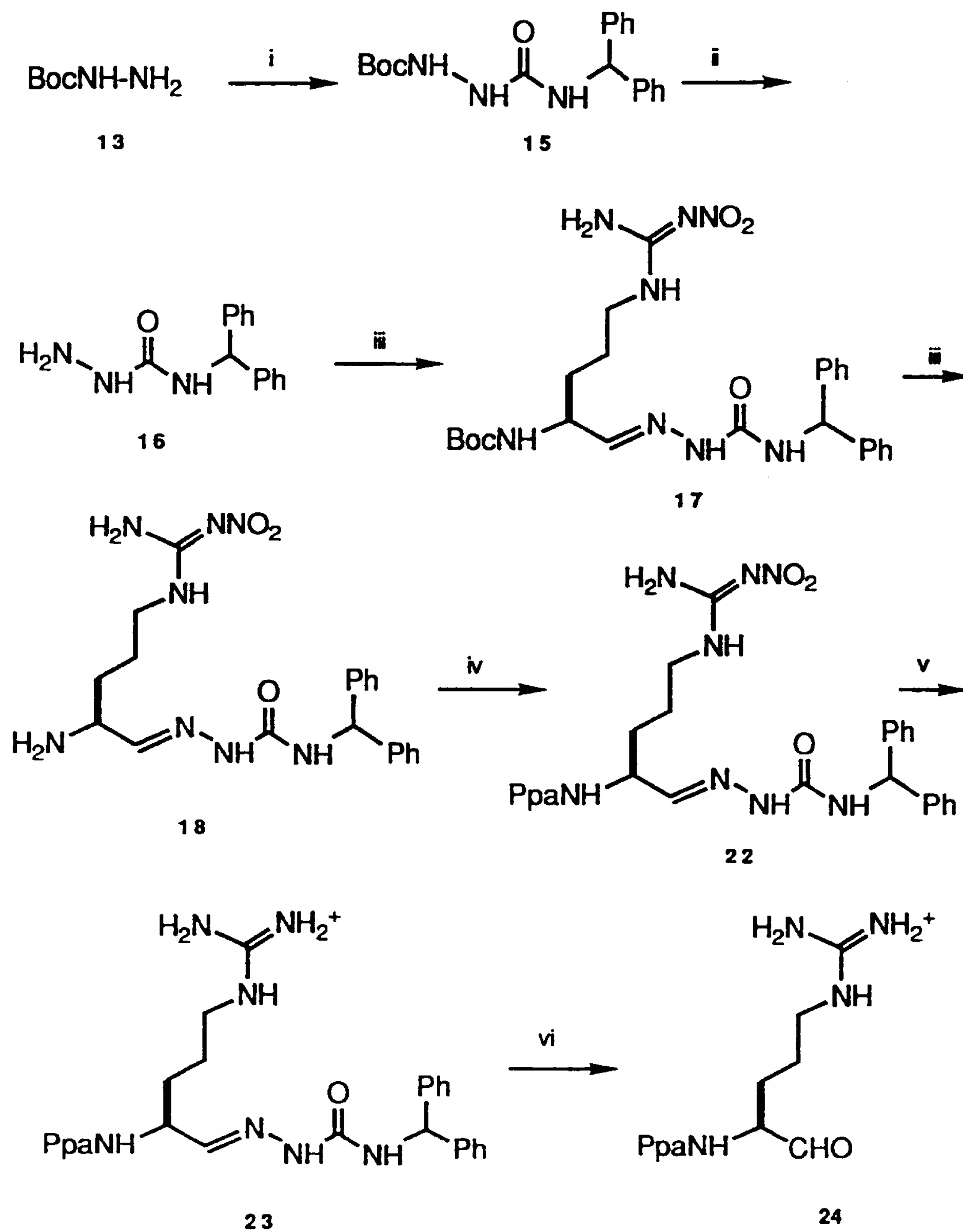


FIG. 3

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/00906

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 37/00; C07C 241/00

US CL : 514/18, 19; 530/331; 562/560

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/18, 19; 530/331; 562/560

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF MEDICINAL CHEMISTRY, Volume 33, Issued 1990, S. Bajusz, "Highly Active And Selective Anticoagulants: D-Phe-Pro-Arg-H, A Free Tripeptide Aldehyde Prone To Spontaneous Inactivation, And Its Stable N-Methyl Derivative, D-MePhe-Pro-Arg-H", pages 1729-1735. See entire article.	1-39
Y	US,A 4,171,299 (Hamburger) 16 October 1979. See entire patent.	1-39
Y	US,A 4,161,522 (Hamburger) 17 July 1979. See entire patent.	1-39

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A document defining the general state of the art which is not considered to be part of particular relevance	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E earlier document published on or after the international filing date	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* &	document member of the same patent family
* O document referring to an oral disclosure, use, exhibition or other means		
* P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

31 March 1993

Date of mailing of the international search report

08 APR 1993

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/00906

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemica et Biophysica Acta, Volume 748, Issued 1983, A.H. Patel et al., "Transition-State Affinity Chromatography Of Trypsin-Like Proteinases With Dipeptidyl Argininal Ligands", pages 321-330. See especially pages 321 and 323.	1-44
A	JOURNAL OF CLINICAL INVESTIGATION, Volume 78, Issued 1986, Niederau et al., "Beneficial Effects Of Cholecystokinin-Receptor Blockade And Inhibition Of Proteolytic Enzyme Activity In Experimental Acute Hemorrhagic Pancreatitis In Mice", pages 1056-1063. See entire article.	1-45
A	Aoyagi et al., PROTEASES AND BIOLOGICAL CONTROL, published 1975 by Cold Spring Harbor Laboratory Press, pages 429-454, especially pages 430-431.	1-45